EXHIBIT 21

original article

Diabetes, Obesity and Metabolism 2013.

A prospective, claims-based assessment of the risk of pancreatitis and pancreatic cancer with liraglutide compared to other antidiabetic drugs

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ORIGINAL

Aim: We evaluated the relationship between liraglutide and acute pancreatitis or pancreatic cancer in an ongoing post-marketing safety assessment programme.

Methods: Initiators of liraglutide, exenatide, metformin, pioglitazone or groups containing initiators of dipeptidyl peptidase-4 inhibitors or sulfonylureas were identified in a US commercial health insurance claims database (1 February 2010 to 31 March 2013) and followed for a median of 15 months. We estimated incidence rates (IR/100 000 person-years), rate ratio (RR) and 95% confidence intervals (CI) of new insurance claims with diagnoses of primary inpatient acute pancreatitis or pancreatic cancer from Poisson regression models.

Results: The IR for acute pancreatitis for liraglutide was 187.5 compared with 154.4 for all non-glucagon-like peptide-1 (GLP-1)-based therapies (adjusted RR 1.10; CI 0.81–1.49). The IR for pancreatic cancer was 19.9 for liraglutide compared with 33.0 for all non-GLP-1-based therapies (adjusted RR 0.65; 95% CI 0.26–1.60).

Conclusion: We did not observe excess risk of either outcome associated with liraglutide relative to individual or pooled comparator drugs.

Keywords: GLP-1, pharmaco-epidemiology, observational study

Date submitted 24 July 2013; date of first decision 13 August 2013; date of final acceptance 29 October 2013

Introduction

Liraglutide is a once-daily glucagon-like peptide-1 (GLP-1) analogue for the treatment of type 2 diabetes. Before FDA approval, we initiated a prospective surveillance programme to evaluate potential adverse effects of liraglutide in the USA. Thyroid cancer is the primary endpoint; however, acute pancreatitis and pancreatic cancer are evaluated in the programme. Recent publications have questioned the pancreatic safety of other GLP-1 receptor agonists (GLP-1RAs) [1–5], thus we performed this interim analysis on acute pancreatitis and pancreatic cancer with liraglutide.

Research Design and Methods

In the surveillance programme, we use a prospective cohort design within the Optum Research Database of national commercial health insurance claims. Accrual is ongoing through 2014. Here we report on all adult initiators (ages 18 and over) of liraglutide or a comparator from 1 February 2010 through 31 December 2012, excluding individuals without medical and pharmacy benefits or less than 6 months of continuous health plan enrollment preceding drug initiation.

Baseline covariates were derived from 6 months of data preceding the date of drug initiation. Follow-up began on the day following initiation and continued until the earliest of insurance disenrollment, claim for acute pancreatitis or pancreatic cancer (separately), or 31 March 2013.

Acute pancreatitis was defined as a hospitalization with an International Classification of Disease, 9th Edition (ICD-9) diagnosis code of 577.0x (positive predictive value 60%) in the primary (first) position on the claim [6]. Pancreatic cancer was defined by an inpatient claim with ICD-9 157.x in the primary position. Individuals with a baseline diagnosis of the outcomes of interest were excluded from the corresponding analysis reported in this article, but not the surveillance programme. Recognizing that early claims for malignancy may represent pre-existing disease, analyses were conducted using all observed pancreatic cancers after drug initiation and, separately, the subset occurring more than 90 days after initiation. We estimated incidence rates (IR/100 000 person-years), rate ratios (RR) and 95% confidence intervals (CI) for liraglutide versus individual and pooled comparators using Poisson regression models. The primary analysis was an 'intention to treat' design in which initiators of a study drug were assumed

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to be on that drug until they experienced a study outcome or were censored. In addition, we conducted an 'as treated' analysis in which exposed person-time was categorized based on observed pharmacy dispensings. In the pooled analysis using metformin, three sulfonylurea therapies, and pioglitazone as a combined comparison group, we excluded exenatide and three dipeptidyl peptidase 4 inhibitors (DPP-4Is), because DPP-4Is and GLP-1RAs have been associated with pancreatic outcomes in previous studies [7,8]. In multivariable Poisson analysis, we controlled for age, gender, healthcare utilization and the Diabetes Complications and Severity Index [9]. We measured healthcare utilization using an index of emergency room visits, diagnoses, inpatient stays, drugs dispensed and physician visits.

Results

Liraglutide initiators were more likely to be women than initiators of all combined comparators (54.2% vs. 49.5%); median age was 53.0 for both groups. Liraglutide initiators had more baseline claims for overweight/obesity (21.2% vs. 13.1%), more indicators of diabetes severity (diabetic neuropathy, nephropathy or retinopathy: 15.7% vs. 8.3%; baseline insulin use: 28.1% vs. 10.3%), and fewer baseline diagnoses of chronic pancreatitis (0.05% vs. 0.14%). Baseline healthcare utilization was generally higher for liraglutide initiators including total costs (median $3235 vs. $1661). The median length of follow-up was 15 months; 29% of the initiators had more than 2 years of follow-up.

The IR per 100 000 person-years of acute pancreatitis for liraglutide was 187.5 compared with 154.4 for pooled comparators (adjusted RR 1.10; 95% CI 0.81–1.49), with rates for individual comparators ranging from 142.4 for metformin to 199.6 for pioglitazone (Table 1). The IR per 100 000 person-years of pancreatic cancer for liraglutide initiators was 19.9 compared with 33.0 for pooled comparators (adjusted RR 0.65; 95% CI 0.26–1.60). Observed IRs for individual comparators ranged from 23.0 for exenatide to 52.9 for the sulfonylureas. The results for both outcomes across individual comparators were similar. Among currently exposed person-time in the 'as treated' analyses, the results were similar (pooled comparators: acute pancreatitis, adjusted RR 1.17, 95% CI 0.86–1.59; pancreatic cancer, adjusted RR 0.40, 95% CI 0.13–1.28).

The median time between drug initiation and initial diagnosis for pancreatic cancer was 270 days across all drugs; 22% of the diagnoses were within the first 90 days. Initiators of liraglutide or exenatide had no early diagnoses, while 7–28% of cancer diagnoses among comparators were within the first 90 days following initiation. Accordingly, in a separate analysis, we excluded diagnoses during the first 90 days of follow-up. The IR among liraglutide initiators remained the same (19.9/100 000 person-years) and for pooled comparators reduced to 25.2/100 000 person-years. The adjusted RR was 0.82 (95% CI 0.33–2.05), with adjusted RRs for individual comparators ranging from 0.52 to 1.06, and none approaching statistical significance.

Table 1. Association between liraglutide and treatment-emergent primary inpatient* acute pancreatitis and pancreatic cancer relative to other specific comparator drugs, and pooled comparator drugs†– intention to treat.

	No. of cases	Person-years‡	IR/100 000 person-years	Adjusted RR, liraglutide versus comparator§	95% CI
Acute pancreatitis					
Liraglutide	47	25 072	187.5		
Pooled comparator drugs, excluding exenatide and DPP-4 inhibitors	472	305 621	154.4	1.10	0.81–1.49
Exenatide (excluding extended release exenatide)	24	13 008	184.5	1.00	0.61–1.63
DPP-4 inhibitors (sitagliptin/saxagliptin/linagliptin)	69	40 364	170.9	1.06	0.73–1.56
Metformin	295	207 177	142.4	1.14	0.83–1.56
Sulfonylureas (glyburide/glipizide/glimiperide)	101	60 361	167.3	1.04	0.73–1.48
Pioglitazone	76	38 083	199.6	0.95	0.65–1.39
Pancreatic cancer					
Liraglutide	5	25 114	19.9		
Pooled comparator drugs, excluding exenatide and DPP-4 inhibitors	101	306,064	33.0	0.65	0.26–1.60
Exenatide (excluding extended release exenatide)	3	13 036	23.0	0.84	0.20–3.52
DPP-4 inhibitors (sitagliptin/saxagliptin/linagliptin)	15	40 424	37.1	0.71	0.25–2.00
Metformin	55	207,458	26.5	0.81	0.32–2.05
Sulfonylureas (glyburide/glipizide/glimiperide)	32	60 443	52.9	0.40	0.15–1.06
Pioglitazone	14	38 163	36.7	0.49	0.17–1.41

IR, incidence rate; CI, confidence interval; RR, relative risk.

*Both outcomes are identified by primary inpatient hospital claims only. Individuals with baseline claims for acute pancreatitis or pancreatic cancer were excluded from the analysis for that outcome.

†Follow-up time for all initiators of the six study drugs/drug combinations began on the day after they initiated the study drug(s) that defines their cohort. Follow-up ended on the earliest of the following: disenrollment from the health plan, primary inpatient claim for acute pancreatitis /pancreatic cancer or 31 March 2013.

‡Person-years vary slightly between the calculations for acute pancreatitis and pancreatic cancer because follow-up time is truncated at the occurrence of the event.

§Factors included in the Poisson regression equation include age, gender, healthcare utilization and Diabetes Complications and Severity Index.

Conclusions

The IRs of health insurance claims representing acute pancreatitis or pancreatic cancer among recipients of liraglutide were similar to those among comparators. The findings for acute pancreatitis are similar to estimates from insurance claims analyses regarding exenatide [4,5,10].

The use of large insurance claims databases permits the rapid study of large numbers of patients under routine care. However, some limitations exist in our analysis regarding outcome ascertainment. The median follow-up time for the study subjects was 15 months. While the length of this period may be sufficient for acute pancreatitis, it may be inadequate for the long-latency outcome of pancreatic cancer. These interim results are based on un-adjudicated diagnoses and the IRs for both outcomes are likely overestimated. Up to 40% of individuals with a primary inpatient diagnostic code for acute pancreatitis will not have a confirmed diagnosis [6]. The accuracy of claims-based pancreatic cancer diagnoses is unclear. Limiting to primary inpatient claims is intended to reduce misclassification resulting from 'rule out' diagnosis or prior history of pancreatic cancer.

Patient attributes may impact the choice of specific therapy. For example, latent pancreatic cancer may affect glycemic control and result in the initiation of newer antidiabetic therapies, including liraglutide [11]. Alternatively, physicians may be less likely to prescribe liraglutide to patients with pancreatitis, a risk factor for pancreatic cancer, because concerns have been raised. Physicians may also monitor users of GLP-1RAs more closely for these outcomes [12], although we observed no claims for pancreatic cancer in the first 90 days of follow-up of liraglutide relative to comparators with up to 28%.

Other methodology issues need to be considered in interpreting these interim findings. There were differences between liraglutide initiators and comparators on several baseline variables. While attempts were made to statistically control for some of these differences, residual confounding may remain. We found similar results using the 'intention to treat' and the 'as treated' approaches, although the 'as treated' analysis may have greater residual confounding because, while exposure status was updated through follow-up, covariate status was not. Similarly, the 'as treated' analysis more strongly assumes that discontinuation of treatment is comparably prognostic for liraglutide and comparators, which is difficult to test.

In summary, we observed no increased risk for acute pancreatitis or pancreatic cancer in association with liraglutide treatment. Future analyses within this data resource will be based on larger cohorts, more follow-up time, and adjudicated outcomes of interest (through review of medical records). Analyses will consider actual treatment patterns in more detail and explore multiple drug combinations.

Acknowledgements

This study was funded by a research contract between Optum and Novo Nordisk A/S. The contract grants Optum oversight of the study conduct, reporting and interpretation, as well as final wording of any resulting manuscripts. Analyses based on part of these data were presented at the NCI-NIDDK workshop on Pancreatitis, Diabetes, and Pancreatic Cancer on 12–13 June 2013. We thank Betsey Gardstein and Heather Norman (Optum) for data analysis and David Dore (Optum and Brown University) for review.

Conflict of Interest

D. F. is an employee of Optum. K. A. C. was an employee of Optum at the time this work was done. H. G., A. M-P. and K. T. are employees of and hold minor portions of employee shares in Novo Nordisk A/S. D. F. participated in the design, conduct, analysis and writing. H. G., K. T. and A. M-P. participated in the writing, K. A. C. participated in the design, analysis and writing.

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EXHIBIT 22

ORIGINAL ARTICLE

Chronic GLP-1 Receptor Activation by Exendin-4 Induces Expansion of Pancreatic Duct Glands in Rats and Accelerates Formation of Dysplastic Lesions and Chronic Pancreatitis in the Kras^{G12D} Mouse Model

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Pancreatic duct glands (PDGs) have been hypothesized to give rise to pancreatic intraepithelial neoplasia (PanIN). Treatment with the glucagon-like peptide (GLP)-1 analog, exendin-4, for 12 weeks induced the expansion of PDGs with mucinous metaplasia and columnar cell atypia resembling low-grade PanIN in rats. In the pancreata of Pdx1-Cre; LSL-Kras^{G12D} mice, exendin-4 led to acceleration of the disruption of exocrine architecture and chronic pancreatitis with mucinous metaplasia and increased formation of murine PanIN lesions. PDGs and PanIN lesions in rodent and human pancreata express the GLP-1 receptor. Exendin-4 induced proproliferative signaling pathways in human pancreatic duct cells, cAMP–protein kinase A and mitogen-activated protein kinase phosphorylation of cAMP-responsive element-binding protein, and increased cyclin D1 expression. These GLP-1 effects were more pronounced in the presence of an activating mutation of Kras and were inhibited by metformin. These data reveal that GLP-1 mimetic therapy may induce focal proliferation in the exocrine pancreas and, in the context of exocrine dysplasia, may accelerate formation of neoplastic PanIN lesions and exacerbate chronic pancreatitis. *Diabetes* 61:1250–1262, 2012

Glucagon-like peptide (GLP)-1 is a proglucagon-derived peptide secreted by gut endocrine cells (L cells) in response to meal ingestion (1). The GLP-1 receptor (GLP-1R) is a G-protein–coupled receptor that is expressed in pancreatic islets and exocrine duct cells (2,3). The increased GLP-1 released after meal ingestion amplifies postprandial nutrient-driven insulin secretion, the so-called incretin effect (4). Based on this property, GLP-1R activation became an attractive therapeutic target for type 2 diabetes mellitus (T2DM).

To overcome the short half-life of circulating GLP-1 that is rapidly degraded by dipeptidyl peptidase (DPP)-4 (5), two strategies have been used in drug development. Oral DPP-4 small molecule inhibitors, such as sitagliptin, prolong the half-life of endogenously secreted GLP-1 (6). Alternatively, GLP-1R peptide agonists given by injection, such as exenatide (7) and liraglutide (8), are resistant to DPP-4 degradation.

Pancreatitis emerged as an unexpected side effect of GLP-1–based therapy in case reports (9,10), and in the U.S. Food and Drug Administration adverse-event reports, liraglutide and sitagliptin showed a signal of pancreatitis (11–13), although analysis of insurance claims records have been reported to show no association between GLP-1–based therapy and pancreatitis (14). Because the human pancreas is inaccessible in treated patients, the question as to whether GLP-1 mimetic therapy acts on the exocrine pancreas has been a subject of animal-based studies.

Pancreatic duct cell proliferation increased transiently with a GLP-1 infusion in Wistar rats (15). Sprague-Dawley rats treated with exendin-4 for 12 weeks developed low-grade chronic pancreatitis (16). Furthermore, DPP-4 inhibition with sitagliptin for 12 weeks was associated with increased pancreatic duct cell replication and acinar-to-ductal metaplasia and, in 1 of 10 rats, chronic pancreatitis (3). However, GLP-1–based therapy also has been reported to not exacerbate chemically induced pancreatitis in mice (17). Also, exenatide was reported to have no effect on ductal turnover in mice or rats, as well as to have a beneficial action in chemically induced pancreatitis (18).

Pancreatic duct glands (PDGs), under conditions of chronic injury, such as chemically induced pancreatitis, may give rise to lesions resembling pancreatic intraepithelial neoplasia (PanIN) (19). To date, there is no information on the actions of GLP-1–based therapy on PDGs or the development of PanIN in pancreata predisposed to dysplasia. Here, we sought to address the following questions. First, does chronic activation of GLP-1Rs by exendin-4 lead to proliferation of the PDGs? Second, is GLP-1R expression present in PDGs and PanIN-like dysplastic lesions? Third, does chronic activation of GLP-1Rs alter the phenotype of Pdx1-Cre; LSL-Kras^{G12D} (Pdx1-Kras) mice?

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Received 9 August 2011 and accepted 16 November 2011.
DOI: 10.2337/db11-1109
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-1109/-/DC1.

See accompanying articles, pp. 986, 989, and 1243.

RESEARCH DESIGN AND METHODS

Rodent studies. All animal studies were approved by the animal use and care committee at the University of California Los Angeles (UCLA). Animals were housed individually in a 12-h light/dark cycle and were weighed weekly to adjust drug doses. Blood glucose and food intake were monitored on a biweekly basis.

Sprague-Dawley rats treated with exendin-4. To establish the actions of GLP-1R activation in the exocrine pancreas, we treated 10 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) with daily injections of 10 μg/kg body wt exendin-4 (ChemPep, Miami, FL) administered by subcutaneous injection for 12 weeks starting at 10 weeks of age (20). Animals were fed chow (Teklad; Harlan Laboratories, Madison, WI) ad libitum. A total

of 15 control rats received daily saline injections. We did not identify PDGs in 5 of 15 controls; therefore, these 5 rats were not included in subsequent analyses. PDGs were identified in all treated rats.

Pdx1-Kras mice treated with exendin-4. To investigate the effect of chronic GLP-1 mimetic treatment on pancreatic cancer precursor lesions, the conditional $Kras^{G12D}$ from Hingorani et al. (21) was used. Experimental animals were generated by crossing Pdx1-Cre with LSL-$Kras^{G12D}$ mice on a C57/BL6 background (both gifts of Guido Eibl, UCLA). Mice (6 weeks old) were fed an AIN-76A–based diet (Research Diets, New Brunswick, NJ) ad libitum for 12 weeks, during which either saline ($n = 7$) or exendin-4 (5 nmol/kg body wt) ($n = 5$) was injected subcutaneously daily.

Pancreas fixation, embedding, and sectioning.

Rat pancreas. After the rats were killed, the rat pancreata were rapidly dissected and then divided into two portions (the head and body of the pancreas and the tail of the pancreas). These were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin, oriented flat to permit subsequent microscopic sections to be made through the longitudinal plane of the pancreas. The block containing the head and body of the pancreas was sectioned at 4 μm intervals to obtain a minimum of 40 sections through the longitudinal plane of the pancreas. A minimum of 10 serial sections were obtained per block from the tail of the pancreas.

Mouse pancreas. Successful excision-recombination events were confirmed by genotyping analysis (Transnetyx, Cordova, TN). Paraformaldehyde fixed, paraffin-embedded pancreatic sections (4 μm) of whole pancreas were stained as below.

Human pancreas. Paraffin-embedded tissue blocks of nonneoplastic human pancreata adjacent to surgically resected pancreatic adenocarcinoma were selected from 10 case subjects from the UCLA pathology archives. All slides and tissue blocks were retrieved after institutional review board approval (no. 11-001724).

Pancreas histology and stains. Tissue sections from rats and mice were deparaffinized in toluene and rehydrated in an ethanol gradient. First, sections were stained in Harris hematoxylin solution (HHS16; Sigma) and eosin Y solution (HT110132; Sigma) to evaluate general histology. PDGs were defined based on the previously described criteria (19) (Fig. 1). Sections were stained by Alcian blue (Electron Microscopy Sciences) and p-aminosalicylic acid (PAS) (Sigma).

For immunohistochemical-immunofluorescent staining, antigen retrieval was performed via microwave heating in citrate buffer (H-3300; Vector, Burlingame, CA), and slides were blocked in Tris-buffered saline (3% bovine serum albumin, 0.2% TX-100, and 2% bovine serum) for 1 h. The following primary antibodies were used for the 12-h incubation (4°C): ductal cell marker cytokeratin (mouse anti-pancytokeratin, 1:50 [Sigma] or rat anti-cytokeratin19/TROMAIII, 1:100 [Hybridoma Bank, University of Iowa, Iowa City, IA]); acinar cell marker amylase (rabbit anti-amylase, 1:300; Abcam); proliferation marker anti–KI-67 (1:50; Dako, Carpinteria, CA); GLP-1R (rabbit anti-human GLP-1R, NLS1206, 1:100; Novus Biologicals, Littleton, CO); and pancreatic and duodenal homeobox-1 (Pdx1) (rabbit anti–Pdx-1, 1:500; β-Cell Biology Consortium, Nashville, TN). Validation of the GLP-1R antisera, NLS1206, was published previously (22). Secondary antibodies labeled with Cy3 and fluorescein isothiocyanate were obtained from The Jackson Laboratories (West Grove, PA) and used at dilutions of 1:100 for the 1-h incubation at room temperature.

For immunohistochemical staining, endogenous peroxidase activity was quenched with 10% methanol, 10% H_2O_2 in Tris-buffered saline, followed by incubation with anti–KI-67 (dilution 1:100; Dako) for 12 h (4°C). Subsequent detection was performed with Envision+ anti-rabbit horseradish peroxidase (Dako), with 3′3′-diaminobenzidine used as the chromogen and hematoxylin as the counterstain.

Likewise, sections of human pancreata were stained with hematoxylin and eosin, Alcian blue, and PAS in order to permit the identification of PDGs and PanIN lesions. Sections adjacent to those with PDGs and PanIN lesions also were stained by immunofluorescence for cytokeratin and for GLP-1R (the same antibodies and dilution as above).

Morphometric analysis of the pancreatic duct gland compartment in rats. The slide with the greatest number of PDGs per animal was selected for quantitative analysis. Slides were digitally scanned in the UCLA Translational Pathology Core Laboratory using an Aperio ScanScope XT (Aperio Technologies, Vista, CA). Quantitative analysis was performed using Aperio Scanscope software. The length of the large duct associated with PDGs and the mean cross-sectional area per PDG were measured in each case. PDGs in the exendin-4–treated rats often showed complex epithelial architecture, including cribriform patterns and pseudosquamous, consistent with epithelial proliferation. The ducts containing this complex PDG epithelial architecture appeared more dilated. To quantify if ductal dilation was present, we measured the longest axis of the large ducts present (duct length) and the inner circumference of the duct lumens. This allowed us to compute a ratio (inner duct luminal circumference to duct length) for each animal.

Histological analysis of chronic pancreatitis and murine PanIN (mPanIN) lesions in Pdx1-Kras mice. Full histologic cross-sections of each pancreas were stained with hematoxylin and eosin for histopathologic examination by two subspecialty gastrointestinal pathologists (D.D. and S.M.D.) blinded to treatment conditions. Chronic pancreatitis was graded using a semiquantitative scoring system, as previously described (23), with slight modification. Chronic pancreatitis was given an index score (0–12) reflecting the sum of scores for acinar loss, lobular inflammation, and fibrosis. Acinar loss was based on the percentage loss across the entire cross-section and graded as 0, absent; 1, 1–25%; 2, 26–50%; 3, 51–76%; and 4, >75%. Inflammation was based on the average number of lobular inflammatory cells per 40× high power field (HPF) (as counted in 10 nonoverlapping HPFs) and graded as 0, absent; 1, 1–30 cells; 2, 31–50 cells; 3, 51–100 cells; and 4, >100 cells. Fibrosis was based on the cumulative area of stromal fibrosis across the entire pancreas and graded as 0, absent; 1, 1–5%; 2, 6–10%; 3, 11–20%; and 4, >20% fibrosis. Duct profiles were evaluated according to established consensus guidelines for the histologic evaluation of mPanIN (24) and quantified as previously described (25). All duct profiles in one full pancreas cross-section were evaluated to determine the relative proportions of nondysplastic (normal, reactive, and metaplastic) ducts and each category of mPanIN lesion. The proportion of each mPanIN lesion to the overall number of duct profiles was recorded for each animal.

Duct cell replication frequency. To determine the frequency of replication of PDG cells and cells in the adjacent main ducts in the head of the pancreas in rats, we quantified the percentage of KI-67–positive cells. Thus, the total number of duct cells (the head of the pancreas) evaluated was 57,261 in exendin-4–treated rats and 61,298 in controls. We also evaluated the frequency of duct cell replication in the sections of the tail of the pancreas immunostained for cytokeratin and KI-67. The total number of duct cells evaluated from the tail was 24,483 in exendin-4–treated rats and 10,706 in controls.

Duct cell replication in pancreata from Pdx1-Kras mice also was analyzed by Ki-67. The extensive acinar-to-ductal metaplasia and frequent dysplastic ductal lesions in GLP-1–treated Pdx1-Kras mice precluded distinguishing replication frequency in the various component of the ductal compartment (PDGs and normal and dysplastic ducts). Slides were analyzed using the Ariol SL-50 automated slide scanner (Leica Microsystems) to quantitate the amount of positive staining for each area of interest containing only ducts and dysplastic ductal tissue. A total number of 121,883 (control group) and 101,830 (exendin-4–treated group) cells were analyzed.

GLP-1 actions on pancreatic duct cells. In vitro experiments were carried out to investigate the effects of exendin-4 on human pancreatic duct epithelial (HPDE) cells (26,27). HPDE cells (kindly made available by Dr. Ming-Sound Tsao, University of Toronto) were maintained in keratinocyte serum-free media supplemented with bovine pituitary extract and human epidermal growth factor (Invitrogen) at 37°C with 5% CO_2. HPDE cells unaffected with the empty vector (pBabepuro) (HPDE-pBP) or with oncogenic pBP-$Kras4B^{G12V}$ (HPDE-Kras) also were used to permit the assessment of GLP-1R activation in the presence of an activating Kras mutation.

To assess the effect of exendin-4 (10 nmol/L) on the phosphorylation of cAMP-responsive element-binding (CREB) protein and the mitogen-activated protein kinases (MAPKs) extracellular signal–related kinase (ERK) 1/2, as well as levels of the cyclin A and D1, 90,000 cells were seeded in six-well plates containing complete medium. At ~70% confluence (day 3 after plating), cells were rinsed with PBS and incubated in starvation medium (lacking bovine pituitary extract and human epidermal growth factor) for 24 h. HPDE cells containing either a control plasmid (pBabepuro; HPDE-pBP) or the mutated oncogenic pBP-$Kras^{G12V}$ (28,29) were pretreated with 100 μmol/L metformin for 30 min. For stimulation experiments, exendin-4 was added in fresh, prewarmed starvation medium for the indicated time points. Stimulation then was stopped by adding ice-cold PBS, and HPDE cells were lysed in lysis buffer (50 mmol/L Hepes, 1% Nonidet P-40, 2 mmol/L Na_3VO_4, 100 mmol/L NaF, 10 mmol/L $PyrPO_4$, 4 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL aprotinin). Protein samples were denatured by boiling at 95°C for 5 min, separated on 4–12% Bis-Tris NuPAGE gels (25–40 μg/lane; Invitrogen), and blotted onto a polyvinylidene fluoride membrane (FluoroTrans; Pall Life Sciences, Ann Arbor, MI). Membranes were probed with the following primary antibodies (dilution for all 1:1,000): rabbit anti-CREB/pCREB; rabbit anti-ERK1/2/pERK1/2 (both Cell Signaling); rabbit anti cyclin A; and rabbit anti–cyclin D1 (both Santa Cruz). After incubation with horseradish peroxidase–conjugated secondary antibody (1:5,000; The Jackson Laboratories), proteins were visualized using enhanced chemiluminescence (Millipore), and levels were quantified using Labworks software (UVP, Upland, CA).

Analytical procedures. Plasma glucose concentrations were measured by the glucose oxidase method (YSI Glucose Analyzer, Yellow Springs, OH). Plasma lipase concentrations were measured by a colorimetric enzyme assay (BioAssay Systems, Hayward, CA).

Statistical analysis. Statistical analysis was performed using the Student *t* test or ANOVA, where appropriate (Statistica, version 6; Statsoft, Tulsa, OK).

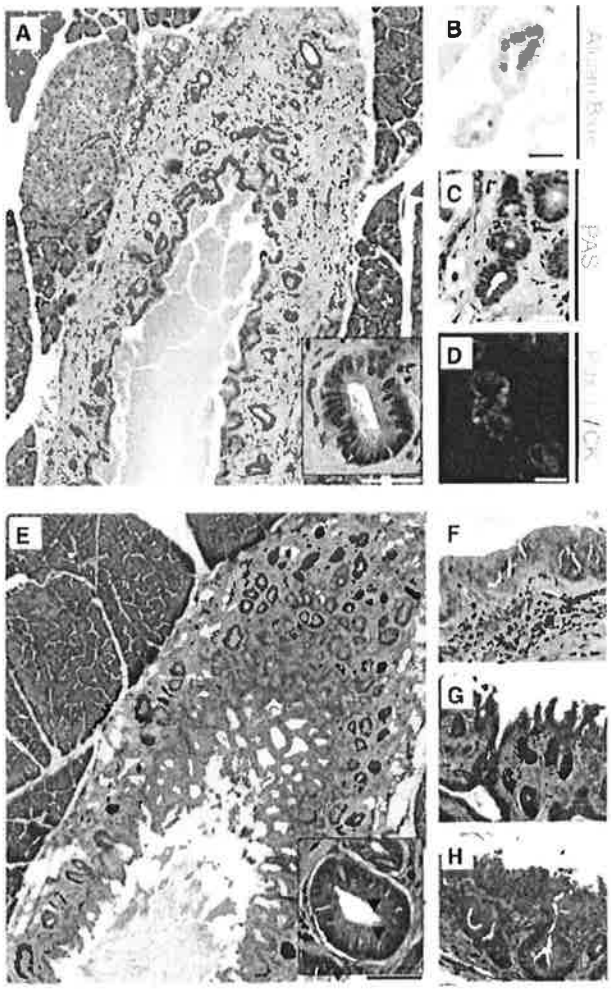

FIG. 1. The extent and frequency of PDGs surrounding the main pancreatic duct are increased by exendin-4 treatment in rats. Sections from the head of the pancreas from an untreated control rat (*A*) and after 12 weeks of daily exendin-4 injections (*E*), in which PDG clusters were identified surrounding the main pancreatic duct. PDGs were confined to the mesenchyme surrounding the main duct in controls but, after exendin-4, expanded to the extent that they projected into the lumen of the pancreatic duct as complex villous-like structures. *A* and *E*, *insets*: PDG cells were columnar in comparison with the cuboidal ductal cells and included goblet-like cells (arrowheads). *B* and *C*: PDGs contained mucin confirmed by Alcian blue and PAS staining. *D*: In contrast to duct cells, PDG cells also expressed Pdx-1 (red; combined staining with the duct cell marker cytokeratin [CK] in green). *E*: PDGs were more common in exendin-4–treated rats (Table 1). *F–H*: In addition, the epithelium often showed pseudostratification and pseudopapillary features, which are features characteristic for PanIN-like lesions. Scale bars = 200 μm (*A* and *E*) and 100 μm (*B–D*), and magnification ×20 (*F–H*). (A high-quality digital representation of this figure is available in the online issue.)

Data in graphs and tables are presented as means ± SEM. Findings were assumed statistically significant at $P < 0.05$.

RESULTS

Metabolic actions of exendin-4 in rats. Twelve weeks of daily exendin-4 injections had the anticipated effects of decreasing weight gain (66 ± 8 vs. 164 ± 5 g; $P < 0.001$ exendin-4 vs. control) and blood glucose levels (99 ± 2 vs. 108 ± 4 mg/dL; $P < 0.01$ exendin-4 vs. control). As expected, exendin-4 decreased daily food intake (153 ± 5 vs. 204 ± 5 mg/day; $P < 0.001$ exendin-4 vs. control), but the treated animals did not seem to be in any apparent pain or distress (Supplementary Fig. 1).

Effects of exendin-4 on exocrine pancreas in rats. Pancreas weight was comparable in the treated versus control group (2.3 ± 0.1 vs. 2.3 ± 0.1 g; exendin-4 vs. control). However, relative to body weight, pancreatic weight in exendin-4–treated animals was increased (0.53 ± 0.02 vs. 0.43 ± 0.02; $P < 0.01$ exendin-4 vs. control) (Supplementary Fig. 1*D*).

There was no histological evidence of pancreatitis in either the exendin-4 or control group. Consistent with this, lipase activity was not changed by exendin-4 (330 ± 19 vs. 299 ± 11 units/L; exendin-4 vs. control) (Supplementary Fig. 1*E*). However, exendin-4 did induce a marked expansion of the PDG compartment (Fig. 1 and Supplementary Fig. 2). PDGs were identified, as previously described, as blind outpouchings from large pancreatic ducts present in the mesenchyme surrounding the ducts. PDG cells were further distinguished from main duct cells by frequently being columnar rather than cuboidal (Fig. 1*A* and *E*, *insets*) and mucin positive (Alcian blue and PAS stains). PDGs also expressed Pdx-1 (Figs. 1*B–D*). There was an ~70% increase in the number of PDGs per unit of length of the main pancreatic duct following exendin-4 treatment (52 ± 7 vs. 31 ± 4 PDGs/mm main duct; $P < 0.05$ exendin-4 vs. control) (Table 1). Moreover, the mean cross-sectional area of individual PDGs still confined within the mesenchyme around the ducts was ~30% increased by exendin-4 treatment (1,184 ± 102 vs. 910 ± 45 μm^2; $P < 0.05$ exendin-4 vs. control), a conservative estimate given that the expanded PDGs adopt a more coiled structure as previously described (19). The latter evaluation also likely underestimates the extent of the expansion of PDGs in exendin-4–treated rats because in many cases the PDGs also expanded into the duct lumen with a complex cribriforming and papillary architecture. To account for this, we quantified the extent to which the main duct lumen was convoluted by any intraluminal projection by computing the ratio of the circumference of the inner duct lumen to the duct length, a metric that was ~35% increased in exendin-4–treated animals (5.0 ± 0.2 vs. 3.7 ± 0.3; $P < 0.01$ exendin-4 vs. control). With exendin-4, the epithelium also showed variable nuclear pseudostratification and loss of polarity, as well as micropapillary architecture (Fig. 1*F–H*), histologic features that can be associated with PanIN lesions and dysplasia when observed in human pancreas, although the implications in rat pancreas are unknown. No carcinoma was seen. Collectively, these findings confirm an increased number of PDGs and expansion of the epithelial cell compartment of both PDGs and large ducts in response to exendin-4 treatment.

The frequency of PDG cell replication was fourfold increased in exendin-4 versus control rats (14.6 ± 3.9 vs. 3.8 ± 0.9%; $P < 0.05$ exendin-4 vs. control) (Fig. 2). The frequency of replication in the main pancreatic ducts was much lower than that in the PDGs but still increased twofold by exendin-4 treatment (5.3 ± 1.8 vs. 2.7 ± 0.6%; $P < 0.05$ exendin-4 vs. control). In contrast, there was no statistically increased frequency of duct cell replication with exendin-4 treatment in the small ducts of the tail of the pancreas (0.62 ± 0.17 vs. 0.42 ± 13%; $P = 0.4$ exendin-4 vs. control).

TABLE 1
Analysis of the PDG compartment

	Control	Exendin-4
Number of PDGs/mm main duct	31 ± 4	52 ± 7*
PDG area (μm^2)	910 ± 45	1,184 ± 102*
Main duct lining-to-length ratio	3.7 ± 0.3	5.0 ± 0.2†

To evaluate the extent of the PDG compartment in treated and control animals, we analyzed the PDG compartment in sections from the head of the pancreas from 10 animals in each group (illustrated in Supplementary Fig. 2). The number of PDGs per millimeter of main duct (first row) and the average size of a PDG (second row) revealed a marked expansion of the PDG compartment after exendin-4 treatment. Furthermore, the main duct appears to be dilated because the ratio of main duct lining to length was increased in the treated group (third row). *$P < 0.05$. †$P < 0.001$.

Actions of GLP-1 mimetic treatment on the exocrine pancreas in the Pdx1-Kras mutant mouse. In Pdx1-Kras mice, 12 weeks of exendin-4 treatment had no impact on body weight (23.2 ± 1.2 vs. 25.8 ± 1.7 g), food intake (18.1 ± 0.7 vs. 19.7 ± 0.5 g per week), or blood glucose levels (83.0 ± 3.4 vs. 76.4 ± 3.7 mg/dL) when compared with littermate control mice. However, GLP-1 mimetic treatment increased pancreatic weight (1.1 ± 0.1 vs. 0.7 ± 0.1 g; exendin-4 vs. control) (Supplementary Fig. 3).

While overall lobular architecture was preserved in both animal groups, the exendin-4–treated animals demonstrated more extensive chronic pancreatitis with greater loss of acini with replacement by reactive or metaplastic duct profiles (Fig. 3). The percentage of pancreas composed of acinar tissue was decreased by 61% by exendin-4 treatment (13.0 ± 13.5% vs. 33.6 ± 14.6%; $P < 0.05$ exendin-4 vs. control). These changes were accompanied by increased inflammation, more extensive stromal fibrosis, and widespread reactive and metaplastic changes, as determined by pancreatitis score (10.0 ± 1.2 vs. 8.6 ± 0.8; $P < 0.05$ exendin-4 vs. control). The plasma lipase activity also was increased with exendin-4 (1,020 ± 164 vs. 678 ± 34 units/L; $P < 0.05$ exendin-4 vs. control) (Supplementary Fig. 3). In comparison to control animals, treated animals showed more extensive acinar-to-ductal metaplasia with replacement of acini by ductules lined by mucin-producing cells primarily with small, round basally oriented nuclei without papillary features (mPanIN1). A minority of the duct profiles demonstrated increased nuclear hyperchromasia and pleomorphism with stratification and micropapillary changes (mPanIN2 and mPanIN3) (Fig. 3). Moreover, GLP-1 mimetic treatment induced increased duct cell proliferation ($P < 0.05$) in Pdx1-Kras mice when compared with control animals (Fig. 4).

GLP-1R expression in PDGs and PanIN lesions. GLP-1R expression was readily detected in pancreatic β-cells in rat and human pancreas, serving as a positive control (data not shown). GLP-1R expression also was present in PDG cells in both rodent and in human pancreas (Fig. 5). GLP-1R expression was not detected in pancreatic acinar cells. GLP-1R expression also was abundantly present in mPanIN lesions

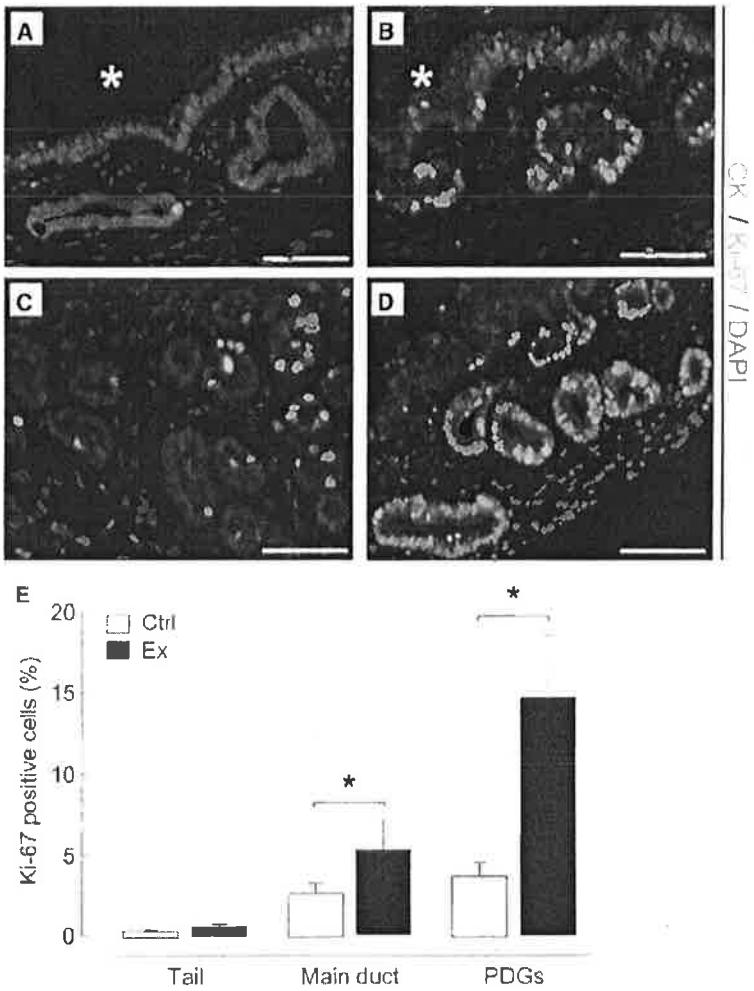

FIG. 2. PDG cell replication is increased by exendin-4 treatment in rats. The frequency of replication ascertained by Ki-67 immunostaining (red; colabeled with cytokeratin [CK] in green) was increased in PDGs compared with adjacent duct cells (*lumen of the large duct) in both control (*A*) and exendin-4–treated (*B*) rats. Replication frequency showed variation within the PDGs in control (*C*) as well as exendin-4–treated (*D*) animals. *E*: However, both the abundance of PDGs and the frequency of replication were increased by exendin-4 treatment. Exendin-4 also increased replication in main duct cells but not in the duct cells in the tail of the pancreas. □, control (Ctrl); ■, exendin-4 (Ex). *$P < 0.05$, scale bars = 100 μm. (A high-quality digital representation of this figure is available in the online issue.)

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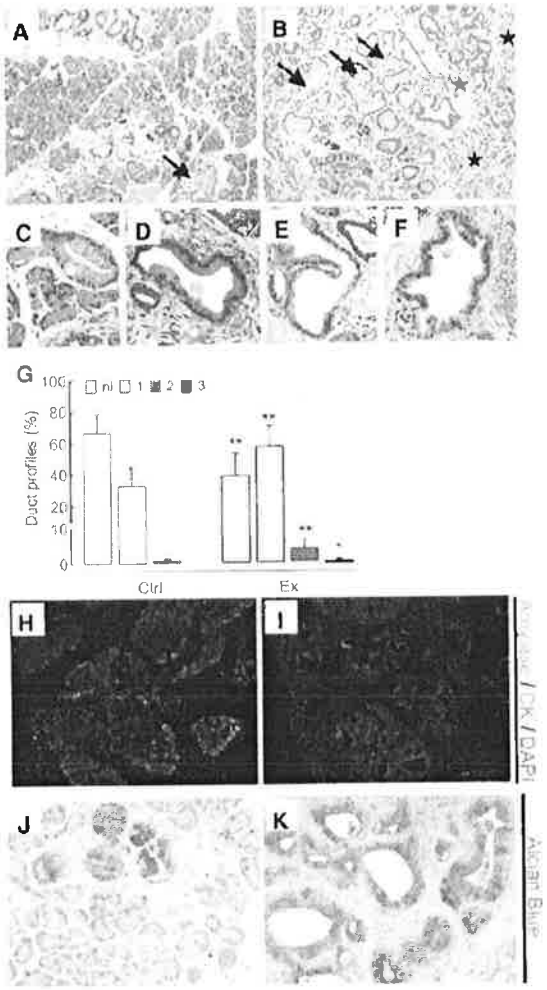

FIG. 3. Exendin-4 treatment increased chronic pancreatitis and the frequency of mPanIN lesions in Pdx1-Kras mice. Pancreata from Pdx1-Kras mice treated for 12 weeks with either vehicle (*A*) or exendin-4 (*B*) (20× objective). The pancreas from the exendin 4–treated animal demonstrates only scant residual intact acini (white arrow) with more extensive inflammation and fibrosis (stars) and more frequent mPanIN (black arrows). *C* and *D*: Low-grade mPanIN1a and mPanIN1b lesions with abundant apical mucin and basally oriented nuclei without significant nuclear pleomorphism or mitotic activity. *E* and *F*: Higher-grade mPanIN2 and mPanIN3 lesions with increased nuclear pleomorphism and focal loss of polarity. *G*) Quantitative analysis of mPanINs showing the percentage of pancreatic ducts with no dysplasia ([]), normal [ni] ; light-grey box, mPanIN1 (1); medium-grey box, mPanIN2 (2); or ■, mPanIN3 (3) lesions in control (Ctrl) and exendin-4 (Ex)–treated mice. *H*: Combined amylase (red) and cytokeratin (CK; green) immunofluorescent staining of the pancreas of a control Pdx1-Kras mouse. *I*: Intact acinar tissue (red) is replaced by cytokeratin-positive (green) ducts, and amylase-positive cells are rarely found in exendin-4–treated animals. Alcian blue staining (blue, counterstained with Nuclear Fast red) reveals mucin-containing lesions in control mice (*J*) and a higher frequency in treated mice (*K*). *P < 0.05; **P < 0.01 vs. control. (A high-quality digital representation of this figure is available in the online issue.)

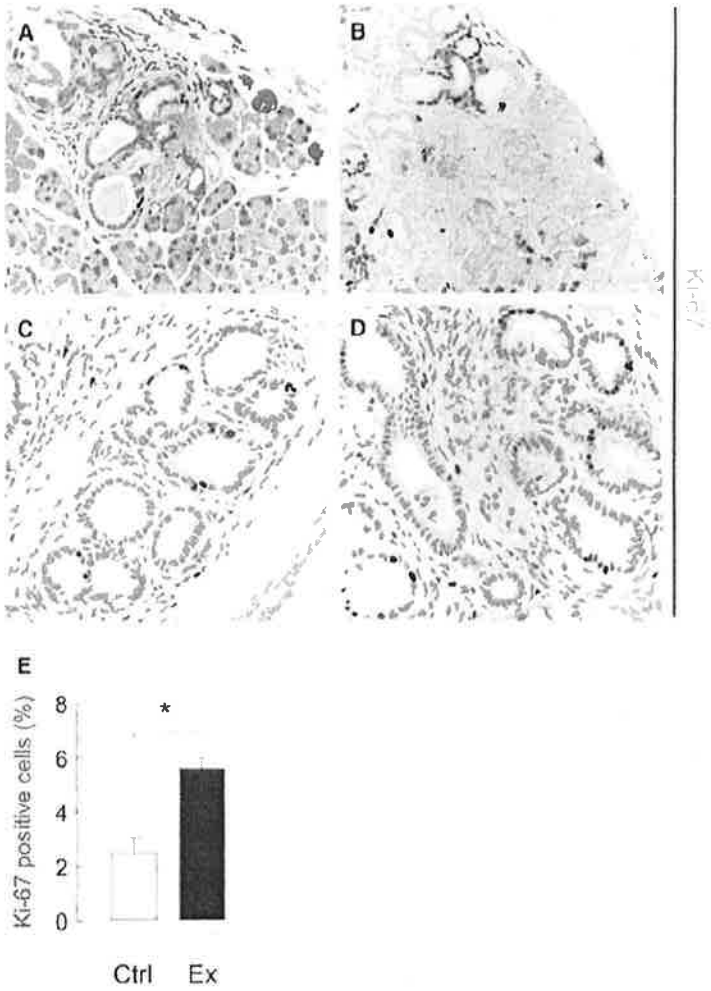

FIG. 4. Duct cell replication frequency is increased in the pancreas of exendin-4–treated Pdx1-Kras mice. Immunohistochemical labeling of Ki-67–positive cells (brown; counterstained with hematoxylin) in benign ducts in areas of intact acinar tissue in control mice (*A*) and exendin-4–treated mice (*B*). An area of ductal proliferation embedded in fibrotic tissue shows an increase in Ki-67–positive cells in the exendin-4–treated group (*D*) compared with controls (*C*). Note the presence of proliferative ducts and mPanIN1a lesion in the exendin-4–treated animal. *E*: Analysis of duct cell proliferation by Ki-67 reveals an increase in the replication frequency in Pdx1-Kras mice treated with exendin-4 (Ex; ■) compared with vehicle control (Ctrl; □). *$P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)

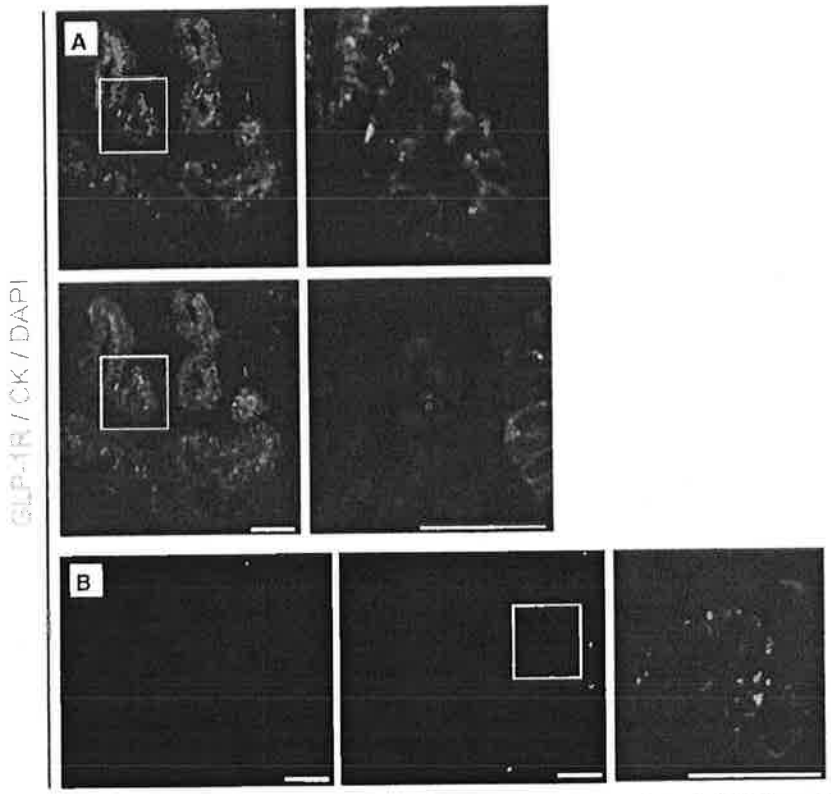

FIG. 5. GLP-1R expression is present in PDGs in rats and humans. *A*: **In the PDGs (shown here for an exendin-4–treated rat), GLP-1R expression (red) was detected by immunofluorescence with combined labeling for the duct cell marker cytokeratin (CK) in green and DAPI to mark the nuclei in blue.** *B*: **Colocalization of GLP-1R and cytokeratin is indicated in the merged images by the color orange. GLP-1R expression was similarly apparent in PDGs in duct cells in the human pancreas. Scale bars = 100 μm. (A high-quality digital representation of this figure is available in the online issue.)**

in the pancreas of Pdx1-Kras mice and humans (Fig. 6). GLP-1R was also detected in areas of acinar-to-ductal metaplasia as well as mPanIN lesions in Pdx1-Kras mice (Fig. 6*A* and *B*). In humans, cells with a columnar phenotype had prominent GLP-1R expression. For example, immunoreactivity was present in PanIN1 lesions but only was minimally detected in adjacent cells with normal cuboidal pancreatic duct morphology in the same duct (Fig. 6*C*). In 6 of 10 human pancreata, GLP-1R expression was detected in a variety of ductal lesions (PanIN1a to PanIN3) (Fig. 6*D* and *E*).

Actions of exendin-4 treatment in human pancreatic duct cells. GLP-1 activation of G-protein–coupled receptors has been reported to activate multiple signaling pathways in pancreatic β-cells, such as the cAMP–protein kinase A and the MAPK pathways leading to phosphorylation of CREB with increased cyclin levels and β-cell replication in pancreatic β-cells (30–32).

To investigate the mechanism of GLP-1–induced duct cell proliferation, we treated HPDE cells with exendin-4 (Fig. 7). CREB phosphorylation increased after 10 min of exendin-4 exposure, reaching a plateau at ~30 min (1.8 ± 0.2-fold vs. control; $P < 0.05$; $n = 3$) (Fig. 7*A*). Exendin-4 induced a time-dependent phosphorylation of the mitogen-activated kinases ERK1 (4.8 ± 0.6-fold) and ERK2 (2.7 ± 0.1-fold, respectively, vs. control at 10 min; $P < 0.01$; $n = 3$) (Fig. 7*B*). Consequently, cyclin D1 protein was induced to a maximum at ~6 h (1.5 ± 0.2-fold vs. control; $P < 0.05$; $n = 3$) (Fig. 7*C*). However, no changes were observed in cyclin

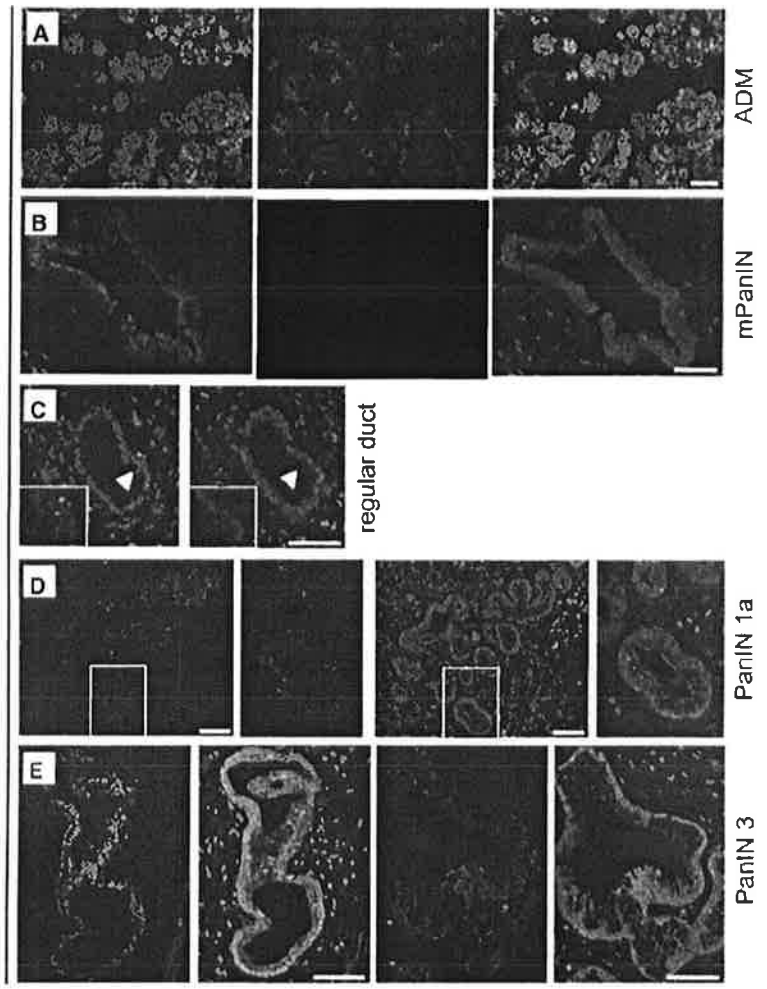

FIG. 6. GLP-1R expression is present in PanIN lesions in Pdx1-Kras mice and humans. GLP-1R (red; shown with combined cytokeratin [CK] labeling in green) was detected in areas of acinar-to-ductal metaplasia (ADM) (*A*) and mPanIN lesion (*B*) in the pancreas of Pdx1-Kras mice. Colocalization of GLP-1R and cytokeratin is indicated in the merged images by the color orange. *C*: In human pancreas, GLP-1R expression was more apparent in the columnar cells (arrowheads) in regular ducts compared with adjacent normal cuboidal duct cells shown away from the arrowhead. *D*: Where duct cells adopt the columnar phenotype (PanIN1a lesion shown), GLP-1R expression becomes more apparent. *E*: In more advanced PanIN3 lesions, GLP-1R immunoreactivity also was clearly present. Scale bars = 100 μm. (A high-quality digital representation of this figure is available in the online issue.)

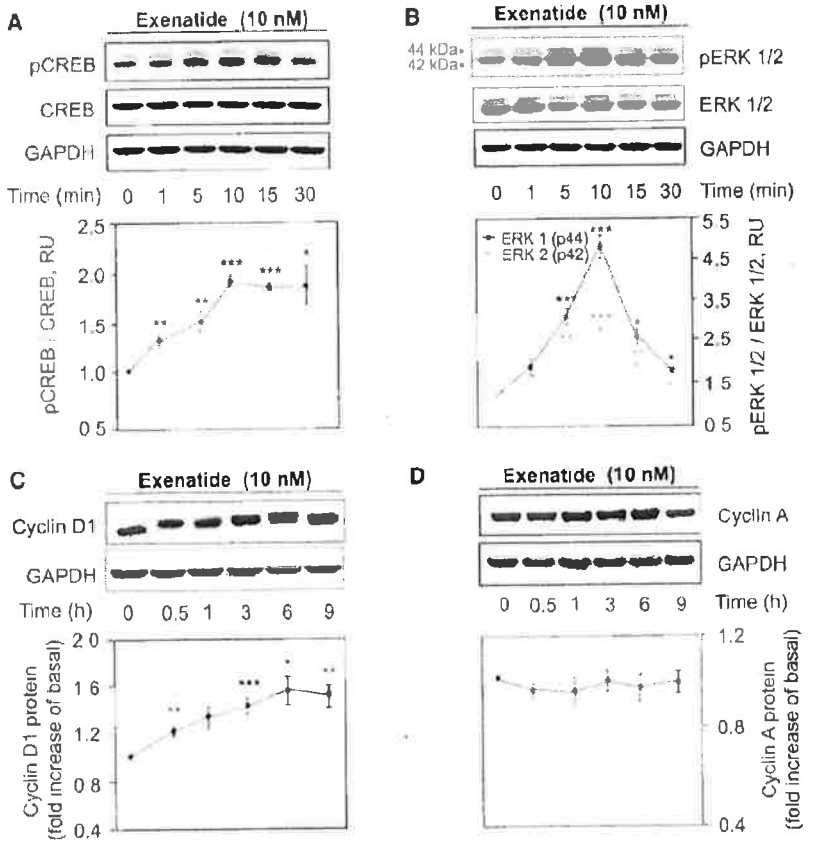

FIG. 7. Exendin-4 actions on human pancreatic duct cells. *A* and *B*: Time-course experiments of CREB (*A*) and ERK1/2 (*B*) phosphorylation in HPDE cells treated with exendin-4 (10 nmol/L) for 0–30 min as indicated. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Representative examples of Western blot experiments are shown in the *top panels* and the corresponding analysis in the *bottom panels*. *C* and *D*: Effect of long-term (0–9 h) stimulation on cyclin D1 (*C*) and cyclin A (*D*) protein levels. Data are expressed as the mean ± SD density ratio of total CREB, ERK1/2 (*A* and *B*), as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*C* and *D*) from 3 to 5 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. untreated control value.

A levels (Fig. 7*D*). We also investigated the actions of exendin-4 with or without metformin in the presence of the activating Kras mutation in HPDE cells. Exendin-4 induced CREB phosphorylation in control (pBP) cells (1.4 ± 0.1-fold pCREB/CREB vs. control; $P < 0.01$; $n = 3$), an effect that was more pronounced in the presence of mutant Kras (1.7 ± 0.1-fold vs. control; $P < 0.001$; $n = 3$), and this effect was abrogated by metformin pretreatment (1.0 ± 0.1-fold vs. control; $n = 3$; $P < 0.01$ vs. exendin-4 treatment alone) (Fig. 8).

DISCUSSION

The possibility that GLP-1 mimetic therapy might induce sustained proliferative changes in the exocrine pancreas is of concern because therapy for T2DM may be administered for decades (33,34). An increased reported adverse event rate in the U.S. Food and Drug Administration adverse-event reporting system for pancreatitis and pancreatic cancer in patients treated with GLP-1–based therapy underscores this concern (35). Because T2DM with obesity is a risk factor for pancreatitis and pancreatic

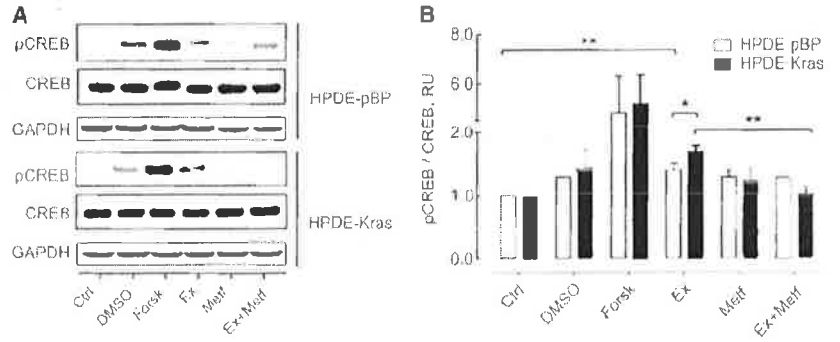

FIG. 8. Oncogenic Kras increases the effects of exendin-4 on human pancreatic duct cells, an effect that is counteracted by metformin. *A*: Representative Western blot of extracts from HPDE cells stably transfected with control vector (pBP) or oncogenic Kras showing CREB phosphorylation at Ser133. Cells were pretreated with metformin (Metf; 100 μmol/L) for 30 min as indicated, prior to a 15-min stimulation with exendin-4 (Ex; 10 nmol/L). Forskolin (Forsk; 10 μmol/L) was used as the positive control. *B*: Statistical analysis shows that phosphorylation of CREB by exendin-4 is higher in HPDE-Kras when compared with HPDE-pBP cells ($P < 0.05$). Metformin treatment abrogated the effect of exendin-4 in HPDE-Kras cells ($P < 0.01$) but not in HPDE-pBP cells. Data are expressed as the mean ± SD density ratio of total CREB from five independent experiments. *$P < 0.05$; **$P < 0.01$. Ctrl, controls DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cancer (36,37), administration of a drug that may further amplify those risks requires closer investigation. In contrast, also unexpectedly, the diabetes medication metformin may decrease the risk of pancreatitis and pancreatic cancer (38,39). Given the recent appreciation that PDGs can give rise to PanIN-like lesions in the context of chronic pancreatitis (19), we first sought to establish the effects of GLP-1R activation on this compartment.

Exendin-4 treatment for 12 weeks induced a marked expansion of the PDG compartment in nondiabetic lean Sprague-Dawley rats. If the pancreas had been sectioned exclusively through the body or tail, no striking abnormalities would have been observed, including no increase in the frequency of replication of duct cells. The normal histology in the most accessible portion of the pancreas and the absence of tumors or overt pancreatitis in lean nondiabetic animals treated with exendin-4 may explain normal exocrine pancreas toxicology screens (40) and some animal studies (17,18). Therefore, to observe the GLP-1–induced changes in PDGs reported in rats here, methodical analysis of the entire pancreas, to include longitudinal sections through the main pancreatic duct, is necessary.

Because PDGs have properties of an adult stem cell compartment (19), it is not surprising that short-term activation of the PDGs by GLP-1 therapy coincident with induced pancreatic injury facilitates recovery from that injury, presumably by fostering regeneration and providing increased protective mucin secretion (17,18). The clinically more relevant question concerns the implication of longer-term stimulation of the PDG compartment and its derivatives.

A total of 12 weeks of exendin-4 therapy in young healthy rats generated mucinous metaplasia and cytologic atypia resembling low-grade PanIN-like lesions in the PDG compartment, features reminiscent of the response to induced chronic pancreatitis in mice and spontaneous chronic pancreatitis in humans (19) (Fig. 1 and Supplementary Fig. 2). However, we also report that GLP-1R expression is present in PDGs and PanIN lesions in rodents and humans, raising the question, does GLP-1 mimetic therapy stimulate the growth of PanIN lesions? Low-grade PanIN lesions are present in 16–80% of normal adult pancreata, the frequency increasing with age (41). PanIN lesions in humans are considered neoplasms and potential precursors for invasive pancreatic cancer based on both pathological findings in humans and longitudinal studies in mice in which mutant Kras is introduced into the pancreas (42). The activating point mutation in the *KRAS* gene is the most frequent mutation present in human PanIN lesions and is considered to be the first step in the progression toward pancreatic cancer (42).

To better appreciate the actions of GLP-1–based therapy in a progression model of PanIN to pancreatic cancer, we treated Pdx1-Kras mice for 12 weeks with exendin-4. Exendin-4 treatment increased duct cell replication, increased the formation of dysplastic mPanIN lesions, and accelerated the development of chronic pancreatitis. These data are consistent with the hypothesis that PanIN lesions contribute to the development of pancreatitis by the obstruction of ductal outflow, with the resulting chronic pancreatitis fostering further development of PanINs (42). The dose of exendin-4 used here, although comparable with that used previously to show the benefit in rodents, exceeds the dose (per kilogram) used in humans (20). A lower dose was used in a recent study to evaluate the effects of exendin-4 on the rodent exocrine pancreas in which no adverse actions were reported (18). However, no data were provided in that report as to whether the dosage of exendin-4 achieved the clinically desired metabolic actions of exendin-4. Moreover, the PDG compartment apparently was not evaluated in those studies, and the animals were not predisposed to dysplasia. It is unknown to date whether a dose of GLP-1 mimetic therapy might be identified that has the intended beneficial actions of enhanced glucose-mediated insulin secretion but no proproliferative effects on the exocrine pancreas.

Evaluation of the proliferative actions of GLP-1 in the exocrine pancreas in humans is not technically feasible. Therefore, we examined the actions of exendin-4 on human pancreatic ductal epithelial cells in vitro. These in vitro studies on the actions of GLP-1R activation in pancreatic duct cells revealed a proproliferative action mediated through the activation of MAPK pathways and phosphorylation of CREB, which was even more apparent in the setting of an activating Kras mutation and inhibited by the actions of metformin. This provides a mechanistic basis for the association of metformin treatment with decreased risk for pancreatitis and pancreatic cancer in individuals with T2DM (38,39). It is also consistent with a previous rodent study in which metformin attenuated the proliferative actions of the DPP-4 inhibitor sitagliptin on the pancreatic ductal tree (3).

In conclusion, we report that treatment of rats for 12 weeks with exendin-4 induced a marked expansion of PDGs through the mechanism of enhanced PDG cell replication. Moreover, we report that the PDGs in rats and humans express GLP-1Rs and that these also are abundantly expressed in PanIN lesions in human pancreas. GLP-1 treatment advances the rate of formation of dysplastic mPanIN lesions and chronic pancreatitis in a mouse model prone to the development of pancreatic ductal adenocarcinoma. Finally, we report that treatment of human pancreatic duct cells with the GLP-1 analog exendin-4 induces proproliferative signaling pathways, an effect that is inhibited by metformin. Collectively, these studies imply that GLP-1–induced proliferation within the exocrine pancreas is focal and may accelerate the development of dysplastic lesions when present.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Grant DK-077967 and the Larry Hillblom Foundation.

No potential conflicts of interest relevant to this article were reported.

B.G. performed the studies and assisted in the study design and interpretation and the writing of the manuscript. A.V.M. assisted in executing the study and study interpretation. D.K. assisted in performing the studies and study interpretation. D.D. and S.M.D. assisted in evaluating the histology, interpreting the study findings, and preparing the manuscript. P.C.B. contributed to the study design, study interpretation, and preparation of the manuscript, and is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors appreciate the technical assistance of Bonnie Yeh and Rosibel Hernandez and the editorial assistance of Bonnie Lui, from the Hillblom Islet Research Center at UCLA. They also acknowledge the provision of the Pdx1-Kras mouse model by Guido Eibl from the UCLA Center for Excellence in Pancreatic Diseases.

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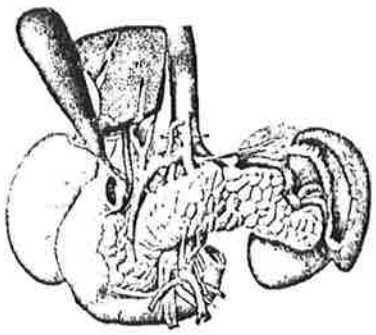

Dipeptidyl Peptidase 4 Inhibitors and Comparative Pancreatic Cancer Risk Among Older Adults

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Introduction

A recent study analyzing human pancreata from 7 sitagliptin-treated patients described potentially detrimental effects of sitagliptin, a dipeptidyl peptidase 4 inhibitor (DPP-4i), on the human pancreas with implications for incident pancreatic cancer.[1] This adds to the concerns already raised by an analysis of the U.S. Food and Drug Administration (FDA) Adverse Events Reporting System (FAERS), which reported increased pancreatic cancer rates with incretin-based antihyperglycemic drugs compared to other antidiabetics.[2] The former study is limited by small numbers, poor matching on baseline characteristics, and absence of information about duration of therapy; and the latter by reporting bias and a lack of a denominator and confounding, among others. Given the lack of population-based studies on this topic, we sought to compare the incidence of pancreatic cancer after initiation of DPP-4i versus sulfonylureas (SU) and thiazolidinediones (TZD). To address concerns about potential outcome detection bias, we compared the cumulative incidence of diagnostic work-up (pancreatic biopsies, abdominal X-rays, computed tomography [CT] scans, laboratory tests) in the two cohorts before and after initiation.

Methods

This study employed a new-user active comparator cohort study design. The study population consisted of patients 65+ years of age initiating DPP-4i, SU, or TZD in a 20 percent random sample of the 2006–2010 Medicare A, B, and D claims data. Prevalent users of the drugs being compared in the 6 months before drug initiation (index date) were excluded. To ensure that patients were actually on the drug, they were required to fill a second prescription of the same drug within 180 days of initiation. Follow-up started at the second fill date. To avoid capturing rule-out diagnoses of pancreatic cancer as the outcome, we defined pancreatic cancer as at least two claims of pancreatic cancer within 2 months. In the primary as-treated analysis, we used propensity score adjusted Cox proportional hazard models to estimate hazard ratios (HR) and 95 percent confidence intervals (CI). Using an active comparator design helped balance the diabetes severity and baseline pancreatic cancer risk in the groups being compared. Diagnostic work-up before and after drug initiation was compared using risk ratios (RR).

Results

In the DPP-4i versus TZD comparison, there were 19,410 DPP-4i initiators, and 23,233 TZD initiators. Over a 9-month median follow-up, 29 DPP-4i initiators had a pancreatic cancer diagnosis. In the DPP-4i versus SU comparison, there were 11,873 and 50,411 DPP-4i and SU initiators, respectively, with 11 pancreatic cancer diagnoses in the DPP-4i group. The hazard of pancreatic cancer with DPP-4i was lower relative to SU (HR = 0.5; CI = 0.3–1.0) and similar to TZD (HR = 1.1; CI = 0.6–1.8). In the 6 months post index, the cumulative incidence of diagnostic procedures among the sitaglitpin initiators (79.4%) was similar to TZD (74.0%) (RR = 1.07; CI = 1.06–1.08) and SU (74.6%) (RR = 1.06; CI = 1.05–1.07). The probability of diagnostic workup pre-index was similar for all groups (~ 80–85%).

Conclusion

Though limited by sample size and duration of exposure, contrary to previous evidence, our data suggest no increased pancreatic cancer risk with DPP-4i relative to SU or TZD and that the probability of diagnostic work-up is not affected by DPP-4i therapy. Analyses including the intent-to-treat approach will be presented.

Funding Source:

National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health. Pilot award through the University of North Carolina's CTSA, UL1TR000083.

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EXHIBIT 24

DIABETES AND CANCER— AN AACE/ACE CONSENSUS STATEMENT

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This document represents the official position of the American Association of Clinical Endocrinologists and the American College of Endocrinology. Where there were no randomized controlled trials or specific U.S. FDA labeling for issues in clinical practice, the participating clinical experts utilized their judgment and experience. Every effort was made to achieve consensus among the committee members. Guidelines are meant to provide guidance, but they are not to be considered prescriptive for any individual patient and cannot replace the judgment of a clinician.

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EXECUTIVE SUMMARY

Epidemiologic data have demonstrated significant increases of various cancers in people with obesity and diabetes. Recently, concern has emerged that antihyperglycemic medications may also be associated with an increased prevalence of multiple cancers; however, available data are limited and conflicting. The American Association of Clinical Endocrinologists (AACE) convened a conference to review factors associated with cancer development in people with obesity and diabetes and to discuss the possible cancer risk of antihyperglycemic medications. Increased body mass index is associated with an increased risk of multiple cancers based on observational epidemiological data, and is closely associated with increased levels of endogenous insulin, insulin-like growth factors, inflammatory cytokines, and other factors that can have downstream pro-cancer growth effects.

The role of hyperglycemia in cancer development is less clear, but an association cannot be ruled out, as current observational data additionally suggest an increased cancer risk in people with diabetes. There is currently insufficient evidence that antihyperglycemic medications may be definitively associated with an increased cancer risk owing to a lack of data from large-scale randomized study designs. Similarly, there is also insufficient evidence showing a positive impact of these medications on cancer development. Clinicians can continue to confidently prescribe all FDA-approved antihyperglycemic medications for the management of hyperglycemia according to established practice guidelines. In patients who have an elevated cancer risk or positive family history of cancer, the cautious selection of antihyperglycemic medications is both prudent and warranted. The AACE additionally advocates for the improved treatment and management of obesity, early cancer screening in patients at increased risk; increased research collaboration, and improved study designs to address outstanding concerns surrounding the diabetes-cancer relationship.

Abbreviations:
AACE = American Association of Clinical Endocrinologists; **BMI** = body mass index; **CI** = confidence interval; **DPP-4** = dipeptidyl peptidase-4; **EMA** = European Medicines Agency; **FDA** = U.S. Food and Drug Administration; **GLP-1** = glucagon-like peptide-1; **HR** = hazard ratio; **IGF** = insulin-like growth factor; **IGFBP** = insulin-like growth factor binding protein; **IR** = insulin receptor; **RR** = relative risk; **T2D** = type 2 diabetes; **TZD** = thiazolidinedione

INTRODUCTION

A conference and writing task force was commissioned by the American Association of Clinical Endocrinologists (AACE) and the American College of Endocrinology to determine the possible roles of obesity, hyperinsulinism, glucose, and diabetes and its therapies in the pathogenesis of cancer. The purpose of this document is to review the available evidence, provide recommendations to practicing clinicians, and highlight research needs.

Contributions of Different Types of Evidence

Basic research provides mechanisms to explain why an agent may increase the risk of cancer. Epidemiological studies can be hypothesis formulating or testing. Observational analytic epidemiological studies are hypothesis testing for moderate to large effects, but hypothesis formulating for small effects which require large-scale randomized evidence. All types of research contribute to a totality of evidence upon which rational clinical decisions for individual patients and policy for the health of the general public can be safely based.

OBESITY AND CANCER

Basic Research

Many proposed biological mechanisms link obesity to cancer development (Fig. 1) through the direct or indirect effects of obesity on insulin and insulin-like growth factor-1 (IGF-1), sex hormones, adipokines, and inflammation (1,2). The collective activation of these individual mechanisms promotes an environment of increased proliferation, inhibited apoptosis, and increased genomic instability (1).

Recent tissue-based breast cancer studies have provided support for hypothetical obesity-related cancer mechanisms in humans (3,4). Breast tissue samples obtained from women undergoing surgery for breast cancer have shown a significant direct correlation between body mass index (BMI) and inflammation ($P<.001$), adipocyte size ($P<.001$), and aromatase expression and activity ($P = .02$) (3). Visceral fat and mammary tissues from obese ovariectomized mice were found to have significantly greater numbers of inflammatory foci ($P<.001$), pro-inflammatory mediators ($P\leq.003$), and aromatase activity ($P<.001$) than samples from other low-fat and high-fat comparator groups (4).

Epidemiologic Studies

Obesity is emerging as a leading avoidable cause of mortality, including cancer mortality. In an analysis of data from 57 prospective cohort studies with approximately 900,000 total participants, BMI was a strong predictor of death above and below the apparent optimum of 22.5 to 25 kg/m^2 (5). The progressive excess mortality for BMI above this range is mainly due to vascular diseases. Median survival (average age at death) is reduced by 2 to 4 years at ages 30 to 45 and 8 to 10 years at ages 40 to 45, which is comparable to the hazard of cigarettes.

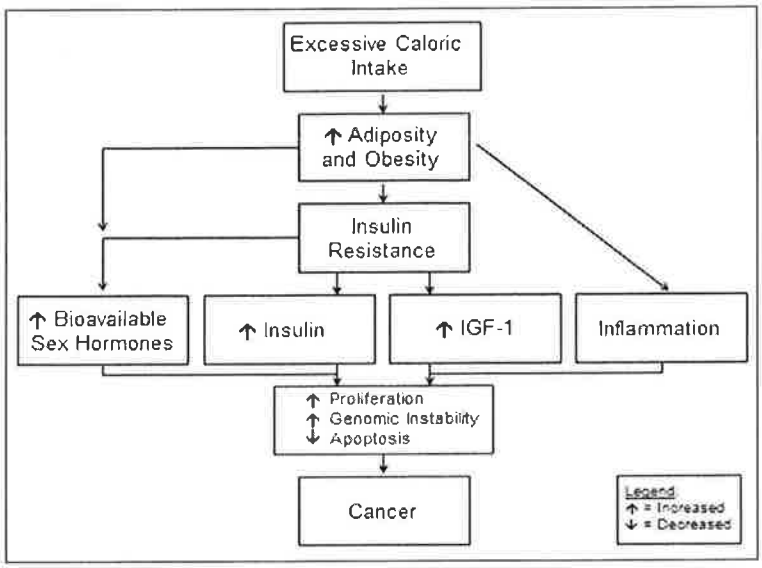

Fig. 1. Biological mechanisms that link obesity with cancer development. *IGF-1* = insulin-like growth factor-1. Adapted from (1).

When compared with overweight or nonobese people, obese individuals or those with a 5-point increase in BMI have a significantly increased risk of many different cancer types (Table 1) (6-10). The strongest associations appear to be for endometrial, gall bladder, esophageal (adenocarcinoma), renal, thyroid, ovarian, breast, and colorectal cancer. Weaker but still statistically significant associations were also observed for leukemia, malignant and multiple melanoma, pancreatic cancer, and non-Hodgkin lymphoma (7,9). Paradoxically, there is some evidence that increased BMI may be protective for lung, esophageal (squamous) (9), and prostate cancer (11) in men, though obesity seems to impart an increased incidence of more aggressive prostate cancers (12). In women, increased BMI may be protective for premenopausal breast and lung cancer (9). In the Swedish Obese Subjects (SOS) prospective controlled intervention trial, obese women undergoing bariatric surgery were observed to have a decreased incidence of cancer compared with controls (hazard ratio [HR], 0.58; 95% confidence interval [CI], 0.44-0.77; $P = .0001$) (13). The same effect was not observed in men (HR, 0.97; 95% CI, 0.62-1.52; $P = .90$).

The observed relationship of elevated cancer risk with increased BMI supports the need to advocate for improved diet, greater physical activity, and early cancer screening in obese patients. Opportunities for educating patients on the obesity–cancer relationship and appropriate lifestyle changes may be possible at the cancer screening visit or following the clinical identification of cancer, when patient health awareness and openness to change are likely to be at higher levels (14-16).

Evidence for the link between obesity and cancer outcomes after diagnosis is less clear. In one cohort of the prospective Cancer Prevention Study II, BMI in the obese range (≥30 kg/m^2) was associated with increased overall cancer mortality compared to normal weight (18.5 to 24.9 kg/m^2) in both men (relative risk [RR], 1.09; 95% CI, 1.05-1.14) and women (RR, 1.23; 95% CI, 1.18-1.29) (17). Increased BMI is associated with worsened outcomes for breast (18-20), colon (21), and aggressive prostate cancer (12), but improved outcomes for renal cell carcinoma (22) and endometrial cancer (23). Furthermore, Adams et al observed decreased mortality (HR, 0.54; 95% CI, 0.37-0.78; $P = .001$) for obesity-related cancers with bariatric surgery in women with a BMI ≥35 kg/m^2 (24).

ROLE OF ENDOGENOUS INSULIN IN CANCER

Insulin, IGF-1 and Cancer Development

Obesity-related hyperinsulinemia may affect cancer development through ligand binding with the insulin receptor and/or by increasing circulating IGF-1 levels (Fig. 2) (2). Circulating IGFs are normally bound by insulin-like growth factor binding proteins (IGFBPs). IGFBP-3 binds almost 90% of circulating IGF-1 and -2. In conditions of prolonged hyperinsulinemia, the activities of IGFBP-1 and -2 are diminished, potentially resulting in increased "free" IGF-1 and -2. Direct relationships among increased obesity (or percentage body fat), increased insulin, and "free" IGF-1 levels have been demonstrated (2,25).

Table 1
Meta-analyses Linking Increased BMI (≥25 kg/m^2) With Cancer Risk

Study group	Cancer evaluated	Risk	95% CI
Druesne-Pecollo et al 2012 (7)	Endometrial (second primary)	RR 1.46[a]	1.17-1.83
	Breast (second primary)	RR 1.14[a]	1.07-1.21
	Breast (contralateral)	RR 1.12[a]	1.06-1.20
Crosbie et al 2010 (6)	Endometrial	RR 1.60[a]	1.52-1.68
Renehan et al 2008 (men) (9)	Esophageal (adenocarcinoma)	RR 1.52[a]	1.33-1.74
	Thyroid	RR 1.33[a]	1.04-1.70
	Colon	RR 1.24[a]	1.20-1.28
	Renal	RR 1.24[a]	1.15-1.34
	Malignant melanoma	RR 1.17[a]	1.05-1.30
	Multiple myeloma	RR 1.11[a]	1.05-1.18
	Rectal	RR 1.09[a]	1.06-1.12
	Leukemia	RR 1.08[a]	1.02-1.14
	Non-Hodgkin lymphoma	RR 1.06[a]	1.03-1.09
	Lung	RR 0.76[a]	0.70-0.83
	Esophageal (squamous)	RR 0.71[a]	0.60-0.85
Renehan et al 2008 (women) (9)	Endometrial	RR 1.59[a]	1.50-1.68
	Gallbladder	RR 1.59[a]	1.02-2.47
	Esophageal (adenocarcinoma)	RR 1.51[a]	1.31-1.74
	Renal	RR 1.34[a]	1.25-1.43
	Leukemia	RR 1.17[a]	1.04-1.32
	Thyroid	RR 1.14[a]	1.06-1.23
	Breast (postmenopausal)	RR 1.12[a]	1.08-1.16
	Pancreatic	RR 1.12[a]	1.02-1.22
	Multiple myeloma	RR 1.11[a]	1.07-1.15
	Colon	RR 1.09[a]	1.05-1.13
	Breast (premenopausal)	RR 0.92[a]	0.88-0.97
	Lung	RR 0.80[a]	0.66-0.97
Schouten et al 2008 (10)	Ovarian (premenopausal)	RR 1.72[b]	1.02-2.89
	Ovarian (Postmenopausal)	RR 1.07[b]	0.87-1.33
Olsen et al 2007 (8)	Ovarian	RR 1.30[c]	1.12-1.50

Abbreviations: BMI = body mass index; CI = confidence interval; RR = relative risk.
[a] Risk values per 5-kg/m^2 increase in BMI.
[b] Multivariate risk, obese (BMI≥30 kg/m^2) versus nonobese (BMI 18.5-23 kg/m^2) patients.
[c] Pooled risk, obese (BMI≥30 kg/m^2) versus nonobese (BMI 18.5-24.9 kg/m^2) patients.

Insulin has multiple effects, depending on its interaction with insulin receptors (IRs), which exist in two major isoforms (IR-A and -B) (26,27). Pro-growth mitogenic effects are elicited through the actions of insulin and IGF-1 binding with the IR-A and IGF-1 receptors, respectively (28,29). The independent role of the IR was confirmed by Zhang et al (30), when downregulation of IRs in LCC6 cells reduced xenograft tumor growth in athymic mice and inhibited lung metastasis. Blockade of the IGF-1 receptor has been associated with decreased growth of breast cancer cells (31,32), while enhanced IGF-1 activity has been associated with decreased susceptibility to chemotherapy (33). Both IR-A and IGF-1 receptors are predominantly located in fetal tissue and in adult cancer cells (34). IRs and IGF-1 receptors are overexpressed in human breast cancers (35-38).

Insulin, Insulin-related Markers, and Cancer Risk

Several study groups have investigated the predictive value of plasma insulin levels for pre- and postmenopausal breast cancer (Table 2), with some conflicting observations (39-42). In a case-control study of 99 premenopausal women with recently diagnosed breast cancer, those in the highest quintile of fasting insulin concentration had a nearly 3-fold increased risk of breast cancer

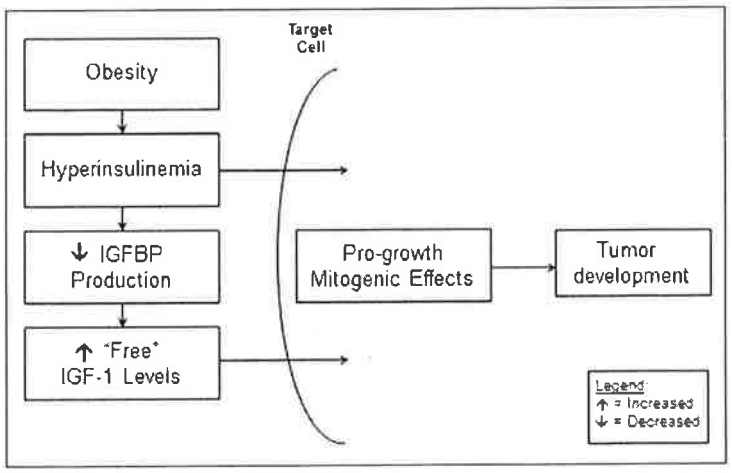

Fig. 2. Obesity and the insulin-IGF-1 hypothesis of cancer development. *IGFBP* = insulin-like growth factor binding protein; *IGF-1* = insulin-like growth factor-1. Adapted from (2).

Table 2
Summary of the Association of Elevated[a] Plasma Insulin, C-Peptide, and IGF-1 Levels With Cancer Risk

Study group	Cancer evaluated	Risk	95% CI
Insulin			
Hirose et al 2003 (41)	Postmenopausal breast cancer	OR 4.48[b]	1.07-18.7
Goodwin et al 2002 (40)	Breast cancer (distant recurrence)	HR 2.0	1.20-3.30
	Breast cancer death	HR 3.1	1.70-5.70
Mink et al 2002 (42)	Breast cancer	RR 1.01[c]	0.55-1.86
Del Giudice et al 1998 (39)	Premenopausal breast cancer	OR 2.83[d]	1.22-6.58
C-Peptide			
Wolpin et al 2009 (47)	Nonmetastatic colorectal death	HR 1.87[e]	1.04-3.36
Pisani et al 2008 (46)	Colorectal	RR 1.35	1.13-1.61
	Breast	RR 1.26	1.06-1.48
	Pancreatic	RR 1.70	1.10-2.63
	Bladder	RR 1.22	1.01-1.47
Ma et al 2004 (44)	Colorectal	RR 2.7[f]	1.20-6.20
IGF-1			
Duggan et al 2013 (43)	Breast cancer (all-cause mortality)	HR 3.10[g]	1.21-7.93
Ma et al 1999 (45)	Colorectal	RR 2.51	1.15-5.46

Abbreviations: CI = confidence interval; HR = hazard ratio; IGF-1 = insulin-like growth factor 1; OR = odds ratio; RR = relative risk.
[a] Defined as values at the highest tertile, quartile, etc.
[b] BMI >23.07, multivariable-adjusted for age, family history, and age at menarche, parity, and at first delivery.
[c] Multivariable-adjusted for age, race, study center, BMI, age at menarche, menopause, and at parity, family history, number of sisters, alcohol intake, and pack-years of smoking.
[d] Multivariable-adjusted for age and weight.
[e] Age-adjusted.
[f] Multivariable-adjusted for BMI, alcohol consumption, vigorous exercise, and aspirin treatment.
[g] Adjusted for BMI, ethnicity, tamoxifen use at time of blood draw, treatment received at diagnosis, and IGFBP-3 levels.

after adjustment compared with those in the lowest quintile (39). Likewise, Hirose et al showed a >4-fold adjusted increased risk of breast cancer in postmenopausal Japanese women with BMI >23.1 kg/m^2 and in the highest tertile of insulin levels compared with the lowest tertile, though not all blood samples were fasting profiles (41). At least one study showed no association of insulin levels with breast cancer risk (42), albeit in a smaller cohort. With respect to distant recurrence and death, Goodwin et al observed that fasting insulin levels in the highest quartile were found to be significantly positively associated in patients with early breast cancer (40).

C-peptide levels and IGF-1 levels have also been linked to cancer risk (43-47). A meta-analysis of 12 epidemiological studies observed that prior to diagnosis, C-peptide or insulin levels at the highest subgrouping were significantly predictive of pancreatic, colorectal, breast, and bladder cancer when compared with lower levels prior to diagnosis (46). Wolpin et al, in a prospective observational study of 373 patients with diagnosed nonmetastatic colorectal cancer, observed a nearly 2-fold higher age-adjusted mortality risk in patients in the top quartile of plasma C-peptide levels compared with those in the lowest quartile (47). Men from the Physician's Health Study in the highest quintile for IGF-1 concentration prior to cancer diagnosis had an increased risk of colorectal cancer compared with those in the lowest quintile (RR, 2.51; 95% CI, 1.15-5.46) (45). Finally, IGF-1 levels and an IGF-1/IGFBP-3 ratio at the highest quintile in women with breast cancer has been observed to confer an approximate 3-fold increased risk of adjusted all-cause mortality compared with patients in the lowest quintiles of these measures (43). Interestingly, clinical trials using humanized monoclonal IGF-1 receptor antagonists to affect cancer outcomes have generally been very disappointing. Besides the suggestive evidence that hyperinsulinemia and obesity are involved in the increased incidence of cancer, other factors, such as leptin, inflammatory cytokines, and reduced sex hormone-binding globulin resulting in more free sex hormones have also been invoked (48).

DIABETES AND CANCER

Animal Models of Diabetes

The independent role of diabetes on cancer development has been difficult to discern, given the fact that obesity is closely associated with inflammation and hyperinsulinemia. Animal studies in transgenic diabetic mice may shed some light on the relative contributions of each of these factors. Models of both skin and mammary carcinogenesis in fatless diabetic (A-ZIP/F-1) mice were found to demonstrate a higher tumor incidence and greater tumor volume than controls in the presence of significantly elevated levels of insulin, IGF-1, growth hormone, and inflammatory cytokines ($P \leq .05$) (49). In a model of murine breast cancer, lean female MKR mice with pronounced diabetes and inactivated IRs and IGF-1 receptors in skeletal muscle were found to have significantly increased insulin/IGF-1 receptor activation in prepubertal mammary gland tissue and increased mammary tumor volume and weight compared with wild-type controls ($P < .05$) (50). Reduced insulin/IGF-1 receptor activation in MKR mice with mammary tumors blocked tumor progression (51). Taken collectively, there appears to be strong support for the interconnected roles of hyperinsulinemia and diabetes in cancer development.

Glucose and Tumor Metabolism

The independent role of hyperglycemia in cancer development is less clear. To achieve growth and proliferation, tumor cells must replicate at higher rates than normal cells, necessitating the need for increased intake of nutrients from the surrounding microenvironment. Glucose is one source of energy for tumor cells to support growth and proliferation. Tumor cells may also rely on the intake of amino acids such as glutamine (52). Glucose uptake is closely regulated by growth factor signaling in normal nonproliferating cells (53); but through genetic mutations, tumor cells can bypass these limitations (52). Activation of growth factor receptors stimulates changes in intracellular signaling, which in turn modify metabolic pathways in support of proliferative growth. Pyruvate kinase isoform M2 (PK-M2) is an example of an enzyme whose activity state is modified to support proliferation in response to changes in intracellular signaling (54). Thus, hyperglycemia is often wrongly implicated as the sole source of cancer nutrition in patients with diabetes, when cancer cells can thrive using other energy sources promoted by genetic mutations and aberrant intracellular signaling.

Diabetes and Cancer Risk

Multiple meta-analyses of case-control and cohort studies have shown that diabetes is associated with a significantly increased risk of breast (55), colorectal (56), endometrial (57), pancreatic (58), and hepatic cancer (59), and non-Hodgkin lymphoma (Table 3) (60). Bladder cancer has also been shown to be positively correlated with diabetes (61), although a recent prospective cohort study of over 170,000 patients indicates that this positive association may be limited to patients with long-standing diabetes (>15 years) or insulin users (62). Prostate cancer risk appears to be decreased in patients with diabetes (63); one possible explanation is that testosterone levels have been shown to be reduced in men with diabetes (64). The conversion of testosterone to dihydrotestosterone promotes prostate cell growth.

Diabetes is also associated with an increase in cancer mortality (Table 4) (65). In the Cancer Prevention II Study, men with diabetes were found to have an increased risk of mortality from hepatic, oropharyngeal, pancreatic,

bladder, colon, and breast cancer and a decreased risk of mortality from prostate cancer (65). In women, diabetes was associated with an increased risk of mortality from breast, hepatic, pancreatic, endometrial, and colon cancer. The findings of the Cancer Prevention II Study are supported by a smaller retrospective cohort study in the United Kingdom of over 8,000 patients with type 2 diabetes (T2D) (66). Two notable discrepant results in the Currie study were the findings of increased prostate cancer mortality and decreased mortality for lung cancer in patients with T2D.

WHAT IS NEEDED FOR CANCER DEVELOPMENT?

After examining the relative contributions of obesity, insulin, IGFs, and diabetes to cancer development, it would appear that the most compelling scenario for cancer development may include a combination of prolonged obesity due to excess caloric intake plus the resulting increase of circulating insulin, IGFs, cytokines, and inflammatory molecules (67). Compelling research in animals has shown that caloric restriction (>10 to 40% of daily intake) can

Table 3
Summary of the Association of Diabetes and Cancer Risk

Study group	Cancer evaluated	Risk	95% CI
Mitri et al 2008 (60)	Non-Hodgkin lymphoma	RR 1.19	1.04-1.35
Friberg et al 2007 (57)	Endometrial	RR 2.10	1.75-2.53
Larsson et al 2007 (55)	Breast	RR 1.20	1.12-1.28
El-Seraq et al 2006 (59)	Hepatic (case-control studies)	OR 2.54	1.82-3.54
	Hepatic (cohort studies)	Risk ratio 2.50	1.93-3.24
Kasper et al 2006 (63)	Prostate	RR 0.84	0.76-0.93
Larsson et al 2006 (61)	Bladder	RR 1.24	1.08-1.42
Huxley et al 2005 (58)	Pancreatic	OR 1.82	1.66-1.89
Larsson et al 2005 (56)	Colorectal	RR 1.30	1.20-1.40

Abbreviations: CI = confidence interval; OR = odds ratio; RR = relative risk.

Table 4
Summary of the Association of Diabetes and Cancer Mortality

Study group	Cancer evaluated	Risk	95% CI
Campbell et al 2012 (men) (65)	Breast	RR 4.20[a]	2.20-8.04
	Hepatic	RR 2.26[a]	1.89-2.70
	Oropharyngeal	RR 1.44[a]	1.07-1.94
	Pancreatic	RR 1.40[a]	1.23-1.59
	Bladder	RR 1.22[a]	1.01-1.47
	Colon	RR 1.15[a]	1.03-1.29
	Prostate	RR 0.88[a]	0.79-0.97
Campbell et al 2012 (women) (65)	Hepatic	RR 1.40[a]	1.05-1.86
	Endometrial	RR 1.33[a]	1.08-1.65
	Pancreatic	RR 1.31[a]	1.14-1.51
	Colon	RR 1.18[a]	1.04-1.33
	Breast	RR 1.16[a]	1.03-1.29
Currie et al 2012 (66)	All cancers	HR 1.09[b]	1.06-1.13
	Breast	HR 1.32[b]	1.17-1.49
	Prostate	HR 1.19[b]	1.08-1.31
	Bladder	HR 1.16[b]	1.02-1.32
	Lung	HR 0.84[b]	0.77-0.92

Abbreviations: CI = confidence interval; HR = hazard ratio; RR = relative risk.
[a] Adjusted for age, education, BMI, smoking, alcohol, vegetable, and red meat intake, physical activity, and aspirin use.
[b] Adjusted for age, sex, smoking status, year of cancer diagnosis, Charlson comorbidity index, Townsend index of deprivation, hemoglobin A_{1C}, and number of general practice contacts.

prevent cancer development (68), with diminished levels of IGF-1 believed to play a central role in mediating this effect (69-71). With tumor cells deriving energy from a variety of sources (glucose and amino acids such as glutamine) and adjusting metabolic pathways to meet homeostatic needs, hyperglycemia may not be an essential component for cancer development in patients with diabetes.

Time from Exposure to Cancer Development

In animal models, the first exposure to a carcinogen causes an "initiating event," whereas genetic damage and consequent DNA repair mechanisms result in fixed genetic mutations (72). Continued exposure to the carcinogen promotes growth of the damaged cell line, resulting in eventual progression to clinical cancer and malignancy. In mice, the time from carcinogen exposure to cancer development is approximately 20 to 50 weeks (73). In humans, this lag time can be as long as 20 to 50 years (74). This is an essential point to consider when weighing the totality of evidence linking disease-state relationships with cancer or the role that pharmacotherapy may play in cancer development.

ANTIHYPERGLYCEMIC DRUGS AND CANCER

Metformin

Metformin use appears to be associated with a neutral-to-decreased effect on cancer incidence and mortality, based on available epidemiological data (Table 5) (66,75-78). A meta-analysis of 13 randomized controlled trials (RCTs) by Stevens et al (78) showed a clinically insignificant 2% increase in the RR of cancer mortality with metformin use in patients with or at risk for diabetes, relative to comparator therapy. The RCTs included in the analysis were not designed a priori to look at cancer incidence but merely reported cancer incidence. Only 9 RCTs looked at metformin monotherapy against a comparator. Other retrospective data point to decreased cancer incidence and mortality in metformin-treated patients (66,75-77). When looking at individual cancer types, metformin use is associated with a significantly lower risk of colorectal, hepatocellular, and lung cancer (77). Nonsignificant lower risks have also been observed for prostate, breast, pancreatic, gastric, and bladder cancer. Overall, metformin has been safely used for the treatment of hyperglycemia for decades. In light of encouraging in vivo and in vitro studies indicating anticancer properties, the use of metformin to improve cancer-related outcomes is actively being investigated in prospective clinical trials (79).

Thiazolidinediones (TZDs)

Evidence from a recent meta-analysis and several observational analytic studies point to a potential concern for increased bladder cancer risk with the use of pioglitazone, particularly with long-term use and large cumulative doses. In a meta-analysis by Colmers et al (80), overall bladder cancer incidence with TZD treatment was 53.1 cases per 100,000 patient-years of treatment. A statistically significant increase in bladder cancer risk was observed when looking at only cohort studies, while a numerically greater but statistically non-significant increase in risk was observed with TZD treatment in RCTs (Table 6). In a similar study, also by Colmers et al (81), TZD use was associated with a decreased risk of colorectal, lung, and

Table 5
Summary of the Association Between Metformin and Cancer Incidence and Mortality

Study group	Outcome	Risk	95% CI
Currie et al 2012 (66)	Cancer mortality	HR 0.85[a]	0.78-0.93
Noto et al 2012 (77)	Cancer incidence	Risk ratio 0.67	0.53-0.85
	Colorectal	Risk ratio 0.68	0.53-0.88
	Hepatocellular	Risk ratio 0.20	0.07-0.59
	Lung	Risk ratio 0.67	0.45-0.99
	Cancer mortality	Risk ratio 0.66	0.49-0.88
Stevens et al 2012 (78)	Cancer mortality	RR 1.02	0.82-1.26
DeCensi et al 2010 (75)	Cancer incidence	RR 0.68	0.52-0.88
	Cancer mortality	RR 0.70	0.51-0.96
Landman et al 2010 (76)	Cancer mortality	HR 0.43[b]	0.23-0.80

Abbreviations: CI, confidence interval; HR, hazard ratio; RR, relative risk.
[a] Adjusted for age, sex, smoking status, cancer diagnosis year, and Charlson comorbidity index.
[b] Adjusted for smoking status, age, sex, diabetes duration, hemoglobin A_{1C}, serum creatinine, BMI, blood pressure, total cholesterol-to-high-density lipoprotein (HDL) ratio, albuminuria, insulin use, sulfonylurea use, and presence of macrovascular complications.

breast cancer. Pioglitazone, but not rosiglitazone, was significantly associated with increased bladder cancer risk (80). These findings are supported by retrospective data indicating that pioglitazone exposure for >24 months or at cumulative doses >28,000 mg is also associated with significantly increased bladder cancer risk (82,83).

When looking at overall cancer incidence in RCTs, there is less concern with TZD use. In the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive) study, there were a total of 97 cases (3.7%) of malignancy reported in the pioglitazone treatment group and 99 cases (3.8%) in the placebo group (84). Of these, 14 cases (0.5%) of bladder cancer were reported with pioglitazone versus 6 cases with placebo (0.2%). After 6 years of observational follow-up of participants in the PROactive study, rates of bladder cancer evened out between the treatment groups (23 cases [0.9%] for pioglitazone versus 22 cases [0.8%] for placebo) (85).

In the Rosiglitazone Evaluated for Cardiovascular Outcomes in Oral Agent Combination Therapy for Type 2 Diabetes (RECORD) trial, rosiglitazone treatment was associated with lower rates of malignancy compared with metformin (0.94 cases per 100 patient-years versus 1.15 cases per 100 patient-years; HR, 1.22; 95% CI, 0.86-1.74) in patients on background sulfonylurea treatment and lower rates of malignancy compared with sulfonylurea (0.93 cases per 100 patient-years versus 1.23 cases per 100 patient-years; HR, 1.33; 95% CI, 0.94-1.88) in patients on background metformin (86). The occurrence of overall malignancy for rosiglitazone, metformin, and glibenclamide in the A Diabetes Outcome Progression Trial (ADOPT) was 1.12, 1.03, and 1.31 cases per 100 patient-years, respectively (86). A meta-analysis of 80 RCTs found no increase in cancer risk with rosiglitazone use relative to comparator groups (odds ratio, 0.91; 95% CI, 0.71-1.16) (87). There is some evidence that TZD use may improve survival in patients with T2D and breast or prostate cancer (88,89).

In summary, TZD-based therapy has been associated with potential cancer risk, primarily pioglitazone with bladder cancer, as well as a protective role (e.g., in colorectal, lung, and breast cancer). Recent data on pioglitazone and bladder cancer essentially removes statistical significance or points to a very small risk leading to bladder cancer. Therefore, clinicians should be confident and continue to use TZDs. However, until more definitive data are available, clinicians should observe and monitor their patients on pioglitazone and follow the U.S. Food and Drug Administration's (FDA) recommendation to not prescribe the drug to people with a history or high risk of bladder cancer.

Incretins

Glucagon-Like Peptide-1 (GLP-1) Receptor Agonists and Thyroid Carcinoma

Prescribing information for GLP-1 agonists includes a cautionary message about preclinical carcinogenicity studies which have shown an increase in thyroid C-cell

Table 6
Summary of TZDs and Cancer Risk

Study group	Analysis groups	Risk	95% CI
Azoulay et al 2012 (82)	Pioglitazone	Rate ratio 1.83[a]	1.10-3.05
	Rosiglitazone	Rate ratio 1.14[a]	0.78-1.68
	Pioglitazone >24 months exposure	Rate ratio 1.99[a]	1.14-3.45
	Pioglitazone >28,000 mg cumulative dosage	Rate ratio 2.54[a]	1.05-6.14
Colmers et al 2012 (80)	TZDs (RCTs)	Risk ratio 1.45	0.75-2.83
	TZDs (cohort studies)	Risk ratio 1.15	1.04-1.26
	Pioglitazone	Risk ratio 1.22	1.07-1.39
	Rosiglitazone	Risk ratio 0.87	0.34-2.23
Colmers et al 2012 (81)	TZDs (colorectal)	Risk ratio 0.93	0.87-1.00
	TZDs (lung)	Risk ratio 0.91	0.84-0.98
	TZDs (breast)	Risk ratio 0.89	0.81-0.98
Lewis et al 2011 (83)	Pioglitazone	HR 1.20[b]	0.90-1.50
	Pioglitazone >24 months exposure	HR 1.40[b]	1.03-2.00

Abbreviations: CI = confidence interval; HR = hazard ratio; RCTs = randomized controlled trials; TZDs = thiazolidinediones.

[a] Adjusted for excess alcohol use, obesity, smoking status, hemoglobin A_{1C}, previous bladder conditions, previous cancer (other than nonmelanoma skin cancer), Charlson comorbidity score, and use of antidiabetic agents at any time.

[b] Adjusted for age, sex, race/ethnicity, smoking status, renal function, bladder conditions, congestive heart failure, income, baseline hemoglobin A_{1C}, diabetes diagnosis at follow-up, duration of diabetes, other cancer prior to baseline, use of antidiabetic medications, and pioglitazone use.

carcinomas in rats (90,91). There are approximately 22- to 45-fold more total C-cells in rodents than in humans, and only rat C-cell lines have been shown to express functional GLP-1 receptors (92). In phase 3 clinical trials, plasma calcitonin, a measure of C-cell hyperplasia and medullary thyroid carcinoma (MTC), did not increase in liraglutide-treated patients and remained below the upper normal ranges for men and women for the duration of the study (92-94). This is in contrast to dose-dependent increases in calcitonin that have been observed in rodents given liraglutide (92). A total of 6 cases of thyroid C-cell hyperplasia have been reported in clinical trials with liraglutide treatment, compared with 2 cases for controls (1.3 cases per 1,000 patient-years versus 1.0 cases per 1,000 patient-years) (90).

A pooled analysis of 19 RCTs by MacConell et al (95) which investigated exenatide BID showed an exposure-adjusted incidence rate of thyroid neoplasms of 0.3 per 100 patient-years compared with zero cases per 100 patient-years for comparators. In an integrated analysis of 10 studies evaluating once-weekly exenatide conducted by the European Medicines Agency (EMA), no cases of MTC were reported (96). While the EMA has currently identified no association between once-weekly exenatide and any malignant neoplasms, future data from ongoing trials and analyses of databases will be monitored.

GLP-1 Receptor Agonists, Dipeptidyl Peptidase-4 (DPP-4) Inhibitors, and Pancreatic Cancer

Based on data gathered from the FDA adverse event databases, GLP-1 receptor agonists and DPP-4 inhibitors may be associated with significantly elevated risks of acute pancreatitis. This has led to speculations about the theoretical possibility of increased incidence of pancreatic cancer (97). However, it is believed that pancreatic tissue requires long-term chronic inflammation to invoke cancer development rather than episodic inflammation due to acute episodes (98,99). In fact, Yachida (100) states that the average time for the development of a pancreatic intraepithelial neoplasia from initiation to the first tumor cell is approximately 12 years, with another 10 years until metastatic pancreatic cancer occurs. Because it has been less than 8 years since the introduction of the first drug in the incretin class (exenatide in 2005), there would not have been enough time for a definitive exposure–cancer development relationship to be established. On the other hand, one cannot exclude the possibility that exposure to these pharmacological classes could theoretically serve as an initiating event or even act to promote an established mutated cell line. From epidemiological data, it is known that the median age of diagnosis of pancreatic cancer is 59 to 64 years, depending on BMI (101). It is possible that patients may have pancreatic cancer without symptoms prior to drug exposure. At this time, no randomized controlled prospective human study of GLP-1 receptor agonists or DPP-4 inhibitors has conclusively shown that these drug classes play a role in the genesis of pancreatic cancer.

Regarding the pancreatitis risk for exenatide, results from two retrospective cohort studies indicate no risk of pancreatitis (102,103), while one study indicates an increased risk for past users but not for recent or current users (Table 7) (104). For sitagliptin, a pooled analysis by

Table 7
GLP-1 Agonists, DPP-4 Inhibitors, and the Risks of Pancreatitis and Pancreatic Cancer

Study group	Risk	95% CI
Acute Pancreatitis: Exenatide		
Dore et al 2011 (104)	Rate ratio (current use) 0.5[a]	0.2-0.9
	Rate ratio (recent use) 1.1[a]	0.4-3.2
	Rate ratio (past use) 2.8[a]	1.6-4.7
Elashoff et al 2011 (97)	OR 10.68	Not given, $P = 2 \times 10^{-16}$
Garg et al 2010 (103)	HR 0.9[b]	0.6-1.5
Dore et al 2009 (102)	RR 1.0	0.6-1.7
Acute Pancreatitis: Sitagliptin		
Garg et al 2010 (103)	HR 0.9[b]	0.7-1.3
Dore et al 2009 (102)	RR 1.0	0.5-2.0
Pancreatic Cancer: Exenatide		
Elashoff et al 2011 (97)	OR 2.95	Not given; $P = 9 \times 10^{-5}$

Abbreviations: CI = confidence interval; DPP-4 = dipeptidyl peptidase-4; GLP-1 = glucagon-like peptide-1; HR = hazard ratio; OR = odds ratio; RR = relative risk.
[a] Propensity score-adjusted.
[b] Adjusted for age, sex, hypertriglyceridemia, alcohol abuse, biliary stone disease, cholestatic liver disease, and drug therapy.

Engel et al (105) of 19 RCTs reported the rate of pancreatitis to be 0.08 events per 100 patient-years versus 0.10 events per 100 patient-years for patients not treated with sitagliptin (difference versus nonexposed, –0.02; 95% CI, –0.20-0.14). Two retrospective cohort studies indicate that sitagliptin has a risk of pancreatitis similar to that of sulfonylureas and metformin (102). Patients taking sitagliptin have the same pancreatitis incidence as control patients with diabetes, at 5.6 cases per 1,000 patient-years (103). There have been postmarketing reports of acute pancreatitis and necrotizing pancreatitis associated with both exenatide and sitagliptin (106,107); however, these events appear to be rare. The use of both DPP-4 inhibitors and GLP-1 receptor agonists is currently discouraged in patients with a history of acute pancreatitis (90,91,108-112).

In March 2013, the FDA released a safety communication stating that the agency was evaluating a new study (113) that suggested an increased risk for precancerous cellular changes in patients with T2D treated with incretin mimetics (114). We added this information for the sake of completeness, although the quality, relevance, and importance of the study are not clear.

In summary, although incretin-based therapies have been associated with a few reports of acute pancreatitis, causal mechanisms have not been established. Moreover, the link to pancreatic cancer is unclear; pathophysiology suggests that a long history of chronic pancreatitis is most likely to be associated with the development of pancreatic neoplasia rather than acute pancreatitis.

Sodium-Glucose Cotransporter 2 (SGLT2) Inhibitors

Within the SGLT2 inhibitor drug class, dapagliflozin, which is not approved in the United States but is approved in Europe, was implicated with an increased incidence of breast and bladder cancer (115). The increased incidence was not statistically significant (116), nor has it been further substantiated. The other members of the class, in particular the now approved canagliflozin, have not shown any cancer signal and are not presently implicated in cancer development (115).

Insulin

Due to the proposed mechanistic association of endogenous hyperinsulinemia with cancer growth and promotion, there is a concern that exogenously administered insulin may amplify the cancer development process. There is evidence from RCTs demonstrating the relative safety of insulin in patients with diabetes with respect to malignancies. The Outcome Reduction with an Initial Glargine Intervention (ORIGIN) study was a RCT that investigated the impact of insulin glargine compared with standard of care for the reduction of cardiovascular outcomes over approximately 6 years of treatment. The rate of cancer incidence was comparable at about 7.6% in both the insulin glargine and standard-care treatment groups (117). Long-term insulin glargine use was not associated with an increased risk of any cancer (HR, 1.0; 95% CI, 0.88-1.13) or cancer death (HR, 0.94; 95% CI, 0.77-1.15) (117), confirming earlier findings by Home et al (118).

Retrospective database analyses provide additional, albeit conflicting, information about the insulin–cancer risk. Insulin treatment alone has been associated with a slightly increased risk of cancer incidence (adjusted HR, 1.44; 95% CI, 1.23-1.67) (119) and cancer mortality (HR, 1.13; 95% CI, 1.01-1.27) (66). However, when looking at patients taking insulin and metformin together, the increased cancer incidence and mortality risks are reduced and are no longer statistically significant (66,120). Cancer risk with insulin therapy has also been observed to rise with an increasing number of yearly prescriptions compared to metformin (120). For insulin glargine, daily doses of 10, 30, and 50 units have been associated with cancer HRs of 1.09 (95% CI, 1.00-1.19), 1.19 (95% CI, 1.10-1.30), and 1.31 (95% CI, 1.20-1.42), respectively, compared with other insulins (121).

There has been recent concern that insulin glargine use may be associated specifically with increased breast cancer risk (122), particularly for patients with T2D and more than 5 years of insulin use (123). More recent studies of large-scale patient databases by the University of North Carolina, Kaiser Permanente of Northern California, and an EMA-commissioned study of Northern European data (124-127), and especially the prospective ORIGIN trial (117), ultimately showed no increased risk of cancer with insulin glargine use, despite previous observational reports of potential increased breast cancer risk. An updated meta-analysis conducted from data in the EMA-commissioned study indicated a summary RR of 0.9 (95% CI, 0.82-0.99) for all cancer and 1.11 (95% CI, 1.0-1.22) for breast cancer (128).

Medications Summary

The contribution of diabetes therapy to cancer development, if at all, appears to be relatively small or nonexistent (Table 8). Prospective clinical studies are not long enough to adequately capture the timeframe of cancer development; thus, it is appropriate for clinicians to remain vigilant based on available evidence. For medications found to be significantly associated with cancer risk, the observed risks or hazards were generally 2-fold or less. Various confounders or poor methodology and study designs may have impacted the observed results. For context, observed risks of 5-fold or higher would represent a signal for safety concerns. For most people with diabetes, the benefits of treatment should take precedence over concerns for potential low-grade cancer risk until more definitive evidence becomes available. The recommendation to consider cancer risk in making medication choices for patients at very high risk of first cancer occurrence or cancer recurrence

Table 8
Summary of Diabetes Medications and Cancer Risk

Medication class	Summary of cancer risk
Metformin	No discernible cancer risk Possible protective benefits on cancer outcomes
TZDs	
Rosiglitazone	No evidence of cancer risk
Pioglitazone	Possible risk of bladder cancer at chronic high doses (>24 months and >28,000-mg cumulative dose)
SGLT2 Inhibitors	No evidence of cancer risk
Incretins	
GLP-1 agonists	No evidence of MTC or pancreatic cancer in humans
DPP-4 Inhibitors	No evidence of MTC or pancreatic cancer in humans
Insulins	Concern of cancer risk at very high doses

Abbreviations: DPP-4 = dipeptidyl peptidase-4; GLP-1 = glucagon-like peptide-1; MTC = medullary thyroid carcinoma; SGLT2 = sodium-glucose cotransporter 2; TZDs = thiazolidinediones.

(129) is prudent. The evidence suggesting a protective effect of metformin and other antihyperglycemic medications against cancer is interesting, but data are limited at this time. Multiple planned and currently ongoing clinical trials may help to shed some light on the protective effects of metformin (79).

IMPLICATIONS FOR PRACTICE

Based on the evidence reviewed, we recommend that healthcare professionals consider the following points for clinical practice:

- Obesity and diabetes are associated with statistically significant and clinically important increased risks of multiple malignancies. This suggests that cancer screening and counseling on lifestyle changes should be a part of regular preventive care in people with obesity and/or diabetes.
- Conversely, individuals who develop "typical" obesity-related cancers, especially at a younger age, should be screened for metabolic abnormalities like insulin resistance, metabolic syndrome, diabetes, and cardiovascular disease.
- Cancer screening tests of proven benefit for malignancies (breast cancer, colon cancer, skin cancer, etc.) in at-risk individuals should begin relatively early. For example, if regular screening for colon cancer starts at age 50, the clinician may consider starting to screen at age 40, as is customary for people with a high risk or family history of colon cancer. Future screenings should be based on current existing recommendations.
- Based on currently understood mechanisms for the development of cancer in obesity and diabetes, proper nutrition management, weight loss, and exercise are equally important to the management of people with cancer as it is to people with obesity and diabetes.
- Several antihyperglycemic medications have been suggested to play a role in the development of certain cancers. The evidence implicating these medications is primarily based on basic research and descriptive epidemiologic studies useful to formulate, not test, hypotheses. To detect reliably the most plausible small to moderate effects requires large-scale randomized evidence. The current totality of evidence should not change clinical practice, though clinicians should be alert to the potential risk and should monitor patients more closely.
- It generally takes many years for cancer to occur clinically, following a complex process of initiation and promotion. Short exposure to any new medication may—but is less likely to—result in clinical cancer development. It is also plausible that the growth of a previously initiated cancer could be promoted by medications.
- At present, the totality of available evidence supports the need for astute clinical judgment in which remote yet plausible cancer risks are weighed against suboptimal glycemic control and higher likelihoods of diabetes complications, especially microvascular, but also macrovascular complications. When prescribing antihyperglycemic medications, a comprehensive risk-benefit analysis must be performed to include an assessment of the baseline personal and familial risk of malignancies in specific organ systems.
- Patients with diabetes undergoing treatment for malignancies should have rigorous and multifactorial approaches to the control of their diabetes. For inpatients, aggressive glycemic management has been associated with improved outcomes.

- There is emerging evidence indicating that metformin and possibly TZDs are associated with lower risks of certain cancers and even may aid as adjunctive therapy in cancer management. Nonetheless, it is premature to prescribe metformin and TZDs solely for these as yet unproven indications.
- The sum of evidence implicating antihyperglycemic medications in the development or promotion of certain cancers is less persuasive. Healthcare professionals should have greater confidence in prescribing all FDA-approved antihyperglycemic medications according to current clinical practice recommendations. Clinicians should exercise caution when choosing medications implicated in the etiology of cancer for patients with the specific organ-related risk.

FUTURE STEPS AND RESEARCH

Given the long duration between exposure to a carcinogen and the development of clinically apparent cancer, large-scale randomized evidence is necessary to detect the most plausible small to moderate effects. A RCT designed to detect a change in risk for overall cancer or a specific cancer, assuming historical rates of occurrence of 1.0 and 0.1%, respectively, would require a total of approximately 25,000 and 250,000 patients, respectively (130). While such trials may be less feasible and too costly, even well-designed observational analytic studies are hypothesis-generating for small to moderate effects.

Multiple questions about the relative contributions of obesity and diabetes to cancer development remain. For instance, what role, if any, does various levels of hyperglycemia play? Do patients with diabetes and controlled glucose levels have a decreased risk of cancer compared with those with uncontrolled glucose levels? It is clear that the basic research in the development of cancers in obesity and diabetes is in its very early stages. Indeed, there is a need for worldwide collaboration, and we call on researchers and academic centers to develop appropriate and needed prospective basic and clinical research.

In light of concerns about diabetes-related medications, future studies should be designed a priori to detect cancer-related outcomes in addition to standard measures of efficacy and safety. Phase 3 randomized trials with longer follow-up times would also be helpful. Greater care and attention to detail are required when communicating scientific data to the community at large and the media. The media should be aware of the implications and potential harms of communicating outcomes without relevant caveats or perspectives.

Obesity is becoming the leading avoidable cause of premature mortality in the world and a leading cause of a variety of health risks, including diabetes and certain cancers; therefore this major risk factor requires preventive and therapeutic interventions. In particular, a focus on children is critical to prevent the further growth of obesity, diabetes, and cancer. Multidisciplinary programs which include basic researchers, epidemiologists, oncologists, endocrinologists, primary care clinicians, and others are critical to understanding and advancing the science.

CONCLUSION

Epidemiology demonstrates a significant increase of cancer in obesity, insulin-resistant states (i.e., metabolic syndrome and polycystic ovary syndrome), and ultimately diabetes. Basic science has suggested plausible mechanisms linking these conditions to the development of cancer. Although medications to treat the hyperglycemia of diabetes have been implicated in increasing the risk of cancer, the totality of evidence is less persuasive, and there is a need for current vigilance and future research. At present, it is necessary to effectively treat hyperglycemia and ensure that the risks of adverse diabetes-related outcomes are minimized in patients. There is currently insufficient evidence to warrant withholding of the use of certain glucose-lowering medications on the basis of cancer concerns. The majority of data linking diabetes medications to cancer arise from meta-analyses of trials not designed to test the hypothesis and observational analytic studies that are subject to bias and confounding. At present, caution and proper monitoring are essential pending the results of RCTs of sufficient size and duration, which are required to minimize the roles of bias, confounding, and chance. It is important to keep in mind that the chronology of cancer development is generally far longer than the time period in which most clinical trials are conducted. The entirety of evidence concerning the interrelationships of obesity, as well as diabetes and its therapies, is incomplete. Further collaborative research between clinicians, including endocrinologists and oncologists, as well as basic, clinical, and epidemiologic researchers, is necessary to complete the evidence on these complex issues.

ACKNOWLEDGMENT

Robert Schupp, PharmD, on behalf of inScience Communications, Springer Healthcare, provided medical writing support funded by AACE.

DISCLOSURES

Chairpersons:

Dr. Yehuda Handelsman reports that he has received research grant support from Boehringer Ingelheim Pharmaceuticals, Inc; Daiichi Sankyo, Inc; GlaxoSmithKline; Merck & Co, Inc; Novo Nordisk, Sanofi-Aventis US LLC; Takeda Pharmaceutical Company USA, Inc; Tolerx, Inc; and XOMA Corp. He has been paid consultant fees by Amarin Corp; Amylin

Pharmaceuticals, LLC; Bristol-Myers Squibb Company; Daiichi Sankyo, Inc; diaDexus, Inc; Genentech, Inc; Gilead; GlaxoSmithKline; Janssen Pharmaceuticals, Inc; Merck & Co, Inc; Sanofi-Aventis US LLC; Santarus, Inc; Tolerx, Inc; VIVUS, Inc; and XOMA Corp. He has also received speakers' bureau honoraria from Amarin Corp; Amylin Pharmaceuticals, LLC; AstraZeneca; Boehringer Ingelheim Pharmaceuticals, Inc; Bristol-Myers Squibb Company; Daiichi Sankyo, Inc; Eli Lilly and Company; GlaxoSmithKline; Janssen Pharmaceuticals, Inc; Novo Nordisk; Santarus, Inc; and VIVUS Inc.

Dr. Derek LeRoith reports that he has received Advisory Board honoraria from AstraZeneca; Bristol-Myers Squibb Company; Merck & Co, Inc; and Sanofi-Aventis US LLC.

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Dr. Samuel Dagogo-Jack reports has received research contracts for his role as principal investigator from AstraZeneca, Novo Nordisk A/S, and Boehringer Ingelheim GmbH; consultant honoraria from Merck & Co, Inc, Santarus and Janssen Pharmaceuticals; and consultant fees from Law Firms Sidley Austin, and Adams & Reese LLP for expert opinion on diabetes.

Dr. Daniel Einhorn reports that he has been an advisor for and holds shares in Freedom Meditech; Halozyme, Inc; and MannKind Corporation. He has received consulting fees for his role as chair of the data management committee for Eli Lilly and Company, for his role as executive committee member on the NAVIGATOR Clinical Trial from Novartis Corporation, and for his role as an advisor to Amylin Pharmaceuticals LLC; AstraZeneca; Bristol-Myers Squibb Company; Eli Lilly and Company; and Novo Nordisk. Dr. Einhorn has also been awarded clinical research grants from Amylin Pharmaceuticals, LLC; AstraZeneca; Eli Lilly and Company; GlaxoSmithKline; Janssen Pharmaceuticals, Inc; Novartis Corporation Novo Nordisk; and Sanofi-Aventis US LLC.

Dr. Alan J. Garber reports that he has received consultant honoraria from Daiichi Sankyo, Inc. and Hoffmann-La Roche, Inc; speakers' bureau and advisory board honoraria from GlaxoSmithKline; Merck & Co, Inc; and Novo Nordisk. He has received clinical research support from Bristol-Myers Squibb Company; GlaxoSmithKline; Merck & Co, Inc; and Novo Nordisk.

Dr. George Grunberger reports that he has received speaker honoraria from AstraZeneca; Bristol-Myers Squibb Company; Eli Lilly and Company; GlaxoSmithKline; Merck & Co, Inc; Novo Nordisk; Sanofi-Aventis US LLC; and Takeda Pharmaceuticals USA, Inc. He has also received research grant support for his role as investigator from Eli Lilly and Company; GlaxoSmithKline; Johnson & Johnson Services, Inc; and Novo Nordisk.

Dr. R. Mack Harrell reports that he has received honoraria from Abbott Laboratories and Veracyte, Inc.

Dr. Robert F. Gagel reports that he has received consultant fees and research grant support for his role as Principal Investigator from AstraZeneca.

Dr. Harold E. Lebovitz reports that he has received consultant fees from AstraZeneca and Bristol-Myers Squibb Company; advisory board honoraria from Amylin Pharmaceuticals LLC and Intarcia Therapeutics, Inc; and advisory board honoraria and shareholder dividends from Merck & Co, Inc.

Dr. Janet B. McGill reports that she has received advisor consulting fees from Abbott Laboratories and LifeScan, Inc; speaker/consultant honoraria from Boehringer Ingelheim Pharmaceuticals, Inc; and speaker honoraria from Bristol-Myers Squibb Company.

Dr. Charles H. Hennekens reports funding from the Charles E. Schmidt College of Medicine at Florida Atlantic University as an independent scientist in an advisory role to the U.S. Food and Drug Administration, U.S. National Institutes of Health, and UpToDate®. He has served as legal consultant for Stryker Corporation, as well as an investigator and sponsor as the chair or a member on data and safety monitoring boards for Actelion Pharmaceuticals, Ltd, Amgen, Inc, AstraZeneca, Bayer, Bristol-Myers Squibb Company, the British Heart Foundation, Cadila, Canadian Institutes of Health Research, Sunovion Pharmaceuticals, Inc, and the Wellcome Foundation. Dr. Hennekens receives royalties for the authorship or editorship of 3 textbooks and as co-inventor on patents concerning inflammation held by Brigham and Women's Hospital. He also has an investment management relationship with the West-Bacon Group within SunTrust Investment Services who has discretionary investment authority.

Funding/Support: The conference, editorial assistance, and the consensus statement were supported by the AACE. Part of the costs of the AACE conference were deferred by grants from Amylin Pharmaceuticals, LLC; Bristol-Myers Squibb Company; AstraZeneca; Eli Lilly and Company; Merck & Co, Inc; Novo Nordisk, Inc; and Sanofi-Aventis, US.

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EXHIBIT 25

DIABETES TECHNOLOGY & THERAPEUTICS
Volume 15, Number 8, 2013

DOI: 10.1089/dia.2013.0177

DTT
Diabetes Technology & Therapeutics

CRITIQUE

An Analysis of Characteristics of Subjects Examined for Incretin Effects on Pancreatic Pathology

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Abstract

A recent autopsy analysis asserted that incretin drugs have the potential of increasing the risk for pancreatic cancer and for pancreatic neuroendocrine tumors. We examined the Network for Pancreatic Organ Donors with Diabetes (nPOD) database from which that analysis was derived. Our findings raise important questions about the comparability of the two groups of diabetes patients used for the analysis. Our review of the data available on the nPOD Web site and our reading of the earlier article lead us to the conclusion that the data, and the implications of the data, as expressed by the authors of the autopsy analysis are vastly overstated and are a misrepresentation of the information available.

Introduction

INCRETIN-BASED THERAPIES for type 2 diabetes mellitus (DM) have been available since the introduction of exenatide, a glucagon-like peptide-1 (GLP-1) receptor agonist, in 2005 and the introduction of sitagliptin, a dipeptidyl peptidase-4 inhibitor, in 2006.[1] Subsequently, several additional drugs in each of these classes have been introduced. These have been shown to be effective glucose-lowering agents that minimize the risk of hypoglycemia.[2] Moreover, meta-analyses suggest that these two classes of agents may have a beneficial effect on cardiovascular outcomes,[3,4] and thus there are large ongoing randomized controlled trials with each of them, designed to determine whether there is indeed a cardiovascular benefit. Both classes of agents are included in current treatment guidelines of the American Diabetes Association and the European Association for the Study of Diabetes[5] and of the American Association of Clinical Endocrinologists.[6] Nonetheless, there have been reports to the U.S. Food and Drug Administration Adverse Event Reporting System of cases of pancreatitis, which has resulted in warnings being added to the label of GLP-1 receptor agonists. The original report and label change may have led to a "notoriety" bias in reporting, as several analyses of insurance company databases have failed to confirm such a risk. The evidence about this has recently been reviewed.[7] Yet, the potential risks of incretin-based therapies have been continually emphasized by Butler and colleagues.[8,9] Recently, Butler et al.[10] presented an autopsy analysis in which they asserted that these drugs have a potential of increasing the risk for pancreatic cancer and for pancreatic neuroendocrine tumors. An accompanying commentary by Kahn[11] raised important questions about the analyses reported. Because the specimens and material came from the Network for Pancreatic Organ Donors with Diabetes (nPOD),[12] an open-access network funded by the JDRF, and because nPOD users have access to the nPOD Web site and may review online both the subject characteristics and the pathology, we decided to review the data in detail, particularly in view of the contrasting commentary by Kahn.[11]

Materials and Methods

The nPOD Web site (http://path-aperio.ahc.ufl.edu) was accessed. Using the case numbers reported by Butler et al.,[10] each case was retrieved, and the data available on the Web site were tabulated. Age at diagnosis was calculated by subtracting duration of DM from age. Although we relied primarily on the information tabulated for each case in the nPOD Web site, a few selected pathology sections were reviewed by one of us (J.L.), a pathologist. The human leukocyte antigen (HLA) types reported were reviewed to determine whether subjects had high-risk type 1 diabetes alleles,[13] reconciling high-risk HLA with current nomenclature.[14] The groupings of subjects were the same as that used by the authors of the original report[10] (i.e., DM subjects treated with incretins [DM-incretin] [$n=8$], DM subjects not treated with incretins [DM-other] [$n=12$], and control subjects without DM [Control] [$n=14$]). Pancreas weight was obtained from Supplementary Table 1 of Butler et al.[10]

Results

Clinical characteristics of individual subjects are shown in Table 1, which is similar in construct to Table 1 of Butler et al.,[10] but our Table 1 includes all fields available on the

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TABLE 1. REVIEW OF CLINICAL AND PATHOLOGICAL FEATURES FROM THE NETWORK FOR PANCREATIC ORGAN DONORS WITH DIABETES DATABASE

Group, case number	Age (years)	Duration (years)	Age (years) at diagnosis	Gender	Race	BMI (kg/m^2)	Treatments	Cause of death	HLA	Auto-antibodies	C-peptide (ng/mL)
DM-incretin group											
6157	74	1	73	F	African American	39	Sitagliptin	ICH/stroke	A*01/02, B*08/51, DR*04/09, DQ*07/09	Negative	2.74
6185	46	15	31	M	African American	41	Sitagliptin, metformin	Anoxia	A*03/74, B*42/53, DR*18/11, DQ*04/06	Negative	26.42
6186	68	5	63	M	White	21	Sitagliptin, metformin	ICH/stroke	A*11/20, B*07/49, DR*07/15, DQ*02/02	Negative	2.98
6189	49	26	23	F	White	36	Exenatide, metformin, glipizide	Stroke	A*11/-, B*27/35, DR*103/-, DQ*05/-	IAA-positive	1.85
6199	53	20	33	M	African American	30	Sitagliptin, insulin	ICH/stroke	A*03/30 B*44/63 DR*01/12 DQ*05/-	Negative	8.87
6194	47	13	34	M	White	24	Sitagliptin, insulin	ICH/stroke	A*02/03, B*55/57, DR*04/07, DQ*08/09	Negative	0.16
6203	68	5	63	M	White	33	Sitagliptin, metformin	Stroke	A*24/33, B*35/65, DR*0/11, DQ*05/07	Negative	6.24
6206	59	10	49	M	White	42	Sitagliptin, metformin	Stroke	A*02/26, B*38/62, DR*04/13, DQ*06/08	Negative	11.15
DM-other group											
6028	33	17	16	M	African American	30	Insulin	Gunshot	A*02/-, B*44/45, DR*14/-, DQ*05/-	Negative	22.4
6059	18	0.3	17.7	F	Hispanic	39	None	CVD	A*02/11, B*35/50, DR*07/08, DQ*02/0	Negative	10.68
6108	57	2	55	M	Asian	30	Metformin	ICH/stroke	A*11/24, B*38/-, DR*15/-, DQ*05/-	Negative	1.25
6110	20	0.2	19.8	F	African American	40	None	ICH/stroke, DKA	A*23/34, B*65/53, DR*18/15, DQ*04/06	Negative	0.58
6109	48	-		F	Hispanic	33	None	ICH/stroke, DKA	A*02/68, B*35/39, DR*14/16, DQ*07/-	mIAA+	<0.05
6114	42	2	40	M	White	31	Metformin (noncompliant)	Asphyxiation	A*03/31, B*07/62, DR*07/15, DQ*06/09	Negative	0.58
6124	62	3	59	M	White	34	Metformin	ICH/stroke	A*29/-, B*27/45, DR*04/15, DQ*06/07	Negative	2.85

HbA1c (%)	*Histopathology*	*β-Cell hyperplasia*	*α-Cell hyperplasia*	*Pancreatitis*	*Amyloid*	*Ductal dysplasia*	*Other*
6.9	Few islets; islet amyloid; no α-cell hyperplasia				Amyloid		
NA	Neuroendocrine tumor; α-cell hyperplasia (follicular); β-cell hyperplasia (follicular)	β-Cell hyperplasia	α-Cell hyperplasia				
NA	Chronic pancreatitis; exocrine atrophy; α-cell hyperplasia (follicular); islet amyloid		α-Cell hyperplasia	Pancreatitis			
NA	Chronic pancreatitis; exocrine atrophy; α-cell hyperplasia (follicular); β-cell hyperplasia (follicular)	β-Cell hyperplasia	α-Cell hyperplasia	Pancreatitis			
NA	Islet amyloid; α-cell hyperplasia (follicular); fibrosis		α-Cell hyperplasia		Amyloid		
7.3	α-Cell hyperplasia; β-cell hyperplasia; PP-cell hyperplasia; acinar atrophy	β-Cell hyperplasia	α-Cell hyperplasia				Renal transplant; acinar atrophy
NA	Exocrine atrophy; α-cell hyperplasia (follicular); β-cell hyperplasia; PP-cell hyperplasia	β-Cell hyperplasia	α-Cell hyperplasia				Exocrine atrophy
8.5	Chronic pancreatitis; ductal hyperplasia; microadenoma pan-tail; α-cell hyperplasia (tail only); β-cell hyperplasia (tail only)	β-Cell hyperplasia	α-Cell hyperplasia	Pancreatitis		Ductal dysplasia	
NA	α-Cell hyperplasia (head, body); some β-cell hyperplasia (tail); few β-cells in head and body; kidney complications	β-Cell hyperplasia	α-Cell hyperplasia		Amyloid		
NA	α-Cell hyperplasia (follicular; especially in tail and body); β-cell hyperplasia (head, body, and tail)	β-Cell hyperplasia	α-Cell hyperplasia				
NA	Islet amyloid (tail); pancreatic stone; no obvious β- or α-cell hyperplasia				Amyloid		
NA	Few β-cells; α-cell hyperplasia (follicular; especially in tail and body)		α-Cell hyperplasia				
8	Islet amyloid; no obvious β- or α-cell hyperplasia (maybe mild for α-cell in tail); PP-cell hyperplasia (head)				Amyloid		
7.8	Islet amyloid; fibrosis; α-cell hyperplasia (follicular; especially tail and body); β-cell hyperplasia follicular in tail); few β-cells in head	β-Cell hyperplasia	α-Cell hyperplasia		Amyloid		
NA	Islet amyloid; hypertrophied islets, some with massive α-cell hyperplasia (tail and body, also follicular in head); some β-cell hyperplasia (tail and body), but few β-cells in head	β-Cell hyperplasia	α-Cell hyperplasia		Amyloid		

(*continued*)

TABLE 1. (CONTINUED)

Group, case number	Age (years)	Duration (years)	Age (years) at diagnosis	Gender	Race	BMI (kg/m^2)	Treatments	Cause of death	HLA	Auto-antibodies	C-peptide (ng/mL)
6127	44	10	34	F	White	30	Insulin	ICH/stroke	A*01/02, B*08/56, DR*04/17, DQ*02/08	mIAA+	0.08
6133	45	20	25	F	White	40	Insulin	CVD	A*02/03, B*37/60, DR*10/15, DQ*05/06	Negative	0.84
6139	37	1.5	35.5	F	Hispanic	45	None	Seizure	A*02/30, B*60/57, DR*04/15, DQ*08/06	Negative	0.6
6142	29	14	15	F	Hispanic	34	None	Bacterial meningitis	A*24/80, B*39/58, DR*14/01, DQ*07/05	mIAA+	0.19
6149	39	20	19	F	African American	29	Insulin	ICH/stroke	A*30/66, B*07/53, DR*09/15, DQ*02/06	GAD+	11.55
Control group											
6009	45	NA		M	White	31	NA	Anoxia	A*11/29; B*50/57, DR*07/15, DQ*06/-	Negative	11.32
6015	39	NA		F	White	32	NA	Anoxia	A*02/02, B*14/51, DR*01/04, DQ*/	Negative	1.99
6012	64	NA		F	White	31	NA	Cerebrovascular/ stroke	A*02/31, B*07/60, DR*15/04, DQ*06/08	Negative	2.97
6016	42	NA		M	White	31	NA	Cerebrovascular/ stroke	A*02/03, B*07/60, DR*13/15, DQ*not tested		NA
6019	68	NA		F	White	24	NA	Head trauma	A*01/03, B*08/35, DR*01/17, DQ*not tested	Negative	0.47
6020	60	NA		M	White	30	NA	Cerebrovascular/ stroke	DRB1*07:01/ 13:01 DQA1* 01:03/ 02:01 ...	Negative	2.82
6022	75	NA		M	White	31	NA	Cerebrovascular/ stroke	A*02/03, B*44/60, DR*04/-, DQ*03/-	Negative	4.99
6034	32	NA		F	White	25	NA	Head trauma	A*03/-, B*07/62, DR*01/08, DQ*05/04	Negative	3.15
6060	23	NA		M	White	33	NA	Head trauma	A*03/24, B*51/39, DR*01/11, DQ*05/07	Negative	13.63

HbA1c (%)	*Histopathology*	*β-Cell hyperplasia*	*α-Cell hyperplasia*	*Pancreatitis*	*Amyloid*	*Ductal dysplasia*	*Other*
NA	Few β-cells, but in big islets (with amyloid?); α-cell hyperplasia (follicular, head, body, and tail)		α-Cell hyperplasia				
NA	Pancreatitis; severe exocrine atrophy; marked islet amyloid; few β- or α-cells and, when detected, in big amyloid-ridden islets; PP-cell hyperplasia (follicular, head)			Pancreatitis	Amyloid		
NA	Pancreatic duct with dysplasia and numerous α-cells (body); some islet amyloid; β-cell numbers appear near normal; marked α-cell hyperplasia (head, body, and tail)		α-Cell hyperplasia		Amyloid	Ductal dysplasia	
NA	β-Cells reduced; moderate acinar atrophy, fibrosis, and ductular dysplasia; some islet amyloid; one lobe (body) with marked β- and α-cell hyperplasia (38712; 38387)		α-Cell hyperplasia		Amyloid	Ductal dysplasia	
NA	Some acinar atrophy; islet amyloid; increased ductal Ki67 (head); few β-cells, possible LADA; α-cell hyperplasia (follicular; head, body, and tail); massive islet in duct cell mass very Glgn+, but some Ins + too (41521 and 41434)		α-Cell hyperplasia		Amyloid		
NA	Numerous islets; seemingly normal β-/α-cell distribution						
NA	Gastric bypass 6 years previously; marked PP-cell hyperplasia in one lobe (46176); odd ductal-like structure with Ins + cells and CD3 infiltrate (14221 and 1458)				Amyloid		Gastric bypass
NA	Chronic pancreatitis (head); many islet β-cells (tail); Ins+ cells in ducts (no α-cell staining available); islets seem OK in head			Pancreatitis			
NA	α-Cell hyperplasia (follicular; tail, 26401); β-cell hyperplasia (follicular; tail, 50711); islets appear normal in head except one lobe where many PP-cells and few β-cells (45608)	β-Cell hyperplasia	α-Cell hyperplasia		Amyloid		
NA	Area of marked α-cell (not β-cell) hyperplasia in head (duct region) 18805, 18932, and 18660; tail seems OK with many islets		α-Cell hyperplasia				
NA	Chronic pancreatitis; fibrosis; acinar atrophy; area of marked α-cell hyperplasia in tail (37252 duct region; 14476 and 14484 Ins adjacent sections); same in head (14224)		α-Cell hyperplasia	Pancreatitis			
NA	Severe acinar atrophy; islet amyloid; few β-cell; many α-cells; indications of α-cell hyperplasia (tail, 57570); β-cell hyperplasia follicular (tail, 45583)		α-Cell hyperplasia		Amyloid		Acinar atrophy
NA	Seems normal						
NA	Pancreatitis, chronic and acute; seems normal; more β-cells than α-cells in most lobes						

(continued)

TABLE 1. (CONTINUED)

Group, case number	Age (years)	Duration (years)	Age (years) at diagnosis	Gender	Race	BMI (kg/m^2)	Treatments	Cause of death	HLA	Auto-antibodies	C-peptide (ng/mL)
6097	43	Preclinical T2D		F	White	36	NA	Cerebrovascular/ stroke	A*02/11, B*44/55, DR*01/14, DQ*05/-	Negative	16.76
6099	14	NA		M	White	30	NA	Head trauma	DR*13/15, DQ*06/06	Negative	5.37
6102	45	NA		F	White	35	NA	Cerebrovascular/ stroke	DR*04/17, DQ*02/0	Negative	0.55
6158	40	NA		M	White	30	NA	Head trauma	A*03/24, B1*49/62, DR*04/13, DQ*06/07	GAD+/ mIAA+	0.51
6165	45	NA		F	White	25	NA	Cerebrovascular/ stroke	A*01/02, B*08/38, DR*13/15, DQ*06/-	Negative	4.45

CVD, cardiovascular disease; DKA, diabetic ketoacidosis; DM, diabetes mellitus; GAD, glutamic acid decarboxylase; Glgn, glucagon; HbA1c, glycated hemoglobin; IAA, insulin autoantibodies; ICH, intracerebral hemorrhage; Ins, insulin; LADA, latent autoimmune diabetes; mIAA, multiple insulin autoantibodies; NA, not available; PP, pancreatic polypeptide producing; T2D, type 2 diabetes.

nPOD Web site and thus provides all available data tabulated on the nPOD Web site. The last six columns summarize key pathological findings, enabling one to see at a glance which subjects had which findings. These findings are taken directly from the Web site and do not involve our own review of the pathology.

Table 2 summarizes by group the frequency of several parameters noted in Table 1 and also includes mean pancreas weight for each group.

It can be seen that there is an 18-year difference in mean age of the two DM groups, with the DM-incretin group having a mean age of 58 ± 4 years and the DM-other group having a mean age of 40 ± 4 years. Moreover, the age range for the DM-incretin group was 46–74 years, whereas the age range for the DM-other group was 18–62 years, with only three subjects in the DM-other group being as old as the youngest subject in the DM-incretin group. Thus, the ages were essentially nonoverlapping. Five of the subjects in the DM-other group were diagnosed prior to 20 years of age, in contrast to none of the subjects in the DM-incretin group. The mean age at diagnosis in the DM-other group was 20.7 years, in contrast to a mean age at diagnosis of 46.1 years in the DM-incretin group. Mean duration of diabetes in the DM-other group was 6.4 years, in contrast to the DM-incretin group, in which it was 11.9 years. Unfortunately, the duration of incretin therapy is not stated on the nPOD Web site. In the DM-incretin group 75% of the subjects were male, whereas only 33% of the DM-other subjects were male. Five of the subjects in the DM-other group were on no diabetes therapy, and four subjects were being treated only with insulin; in the DM-incretin group, seven subjects were treated with sitagliptin, one subject was treated with exenatide, and two of the subjects also were being treated with insulin. Two subjects in the DM-other group died in diabetic ketoacidosis. Only one (13%) of the DM-incretin subjects had a diabetes autoantibody, in contrast to four (33%) of the DM-other subjects. High-risk HLA was present in two subjects in the DM-incretin group (subject #6194 and subject #6206) and one subject in the DM-other group (subject #6127). In total, three (38%) subjects in the DM-incretin group had either high-risk HLA or autoantibodies, whereas in total seven (58%) subjects in the DM-other group had either high-risk HLA or autoantibodies or age at diagnosis of less than 20 years, and an additional subject (diagnosed at 25 years of age) had been treated only with insulin, raising the possibility that as many as 67% of subjects in the DM-other group could have had type 1 DM.

Pancreas weight differed between the groups, as seen in Table 2. However, it should be noted that seven (50%) of the subjects in the control group without DM did not have their

TABLE 2. TABULATION OF DIFFERENT FEATURES AMONG THE THREE GROUPS

	Group		
	DM-incretin	DM-other	Control
n	8	12	14
Age (years)	58	40	45
DM mean (years)	46.9		
Gender	6 M:2 F	4 M:8 F	7 M:7 F
HLA T1D	2 (25%)	1 (8%)	1 (7%)
Islet autoantibodies	1 (13%)	4 (33%)	1 (7%)
T1D HLA or ABS	3 (38%)	4 (33%)	1 (7%)
T1D HLA or ABS or age at diagnosis <20 years	3 (38%)	7 (58%)	1 (7%)
Pancreas weight (g)	113.3	79.3	91[a]
β-Cell hyperplasia	5 (63%)	4 (33%)	3 (21%)
α-Cell hyperplasia	7 (88%)	9 (75%)	6 (43%)
Pancreatitis	3 (38%)	1 (8%)	4 (29%)
Islet amyloid	2 (25%)	9 (75%)	3 (21%)
Ductal dysplasia	1 (13%)	2 (17%)	2 (14%)

[a]Weight not available for seven patients.

ABS, antibodies; DM, diabetes mellitus; F, female; HLA, human leukocyte antigen; M, male; T1D, type 1 diabetes.

HbA1c (%)	*Histopathology*	*β-Cell hyperplasia*	*α-Cell hyperplasia*	*Pancreatitis*	*Amyloid*	*Ductal dysplasia*	*Other*
7.1	Exocrine atrophy; Interesting area of β- and α-cell hyperplasia in body (22825 and 22887) and in tail; islet numbers low in head	β-Cell hyperplasia	α-Cell hyperplasia				Preclinical T2D
NA	Some pancreatitis; CD3+ infiltrates; islets seem normal			Pancreatitis			CD3 infiltrate
6.1	Islets mostly OK except for interesting area of β- and α-cell hyperplasia in tail (23982 and 23638)	β-Cell hyperplasia	α-Cell hyperplasia				
5.6	Exocrine atrophy; mild ductular dysplasia; focal chronic pancreatitis; massive PP-cell hypertrophy (head); few β- and α-cells there; few islets on body and tail			Pancreatitis		Ductal dysplasia	PP staining
5.6	Ductular dysplasia and metaplasia in head and tail (many Ins+ cells there); islets seem OK otherwise					Ductal dysplasia	

pancreas weight noted, thus making the control group number unreliable.

Based on the descriptions on the nPOD Web site, β-cell hyperplasia was more often noted in the DM-incretin group, being found in five (63%) subjects in comparison with four (33%) of the DM-other subjects. In contrast, α-cell hyperplasia was noted in a large number of subjects in both groups with diabetes: seven (88%) DM-incretin subjects and nine (75%) DM-other subjects. Pancreatitis was noted to be present in three (38%) DM-incretin subjects and only one (8%) DM-other subject but was also noted in four (29%) control subjects. Islet amyloid was noted in only two (25%) DM-incretin subjects but in nine (75%) DM-other subjects. Ductal dysplasia was noted more or less equally in all three groups.

Discussion

There is a clear and unambiguous difference between the DM-incretin and DM-other groups. Butler et al.[10] asserted that "pancreata were also obtained from 14 non diabetic (ND) controls matched by age, sex and BMI [body mass index] with the two DM treatment groups" and in the abstract asserted "examination of pancreata from age matched organ donors with type 2 diabetes (DM) treated by incretin therapy ($n=8$) or other therapy ($n=12$) and non diabetic controls ($n=14$)." Although it is true that if you add the two DM groups together the mean age is 46.9 years, versus 45 years in the control group, most of the comparisons and the thrust of the article was comparison of the two DM groups, which were not matched for age, with mean age being 40 years in the DM-other group and 58 years in the DM-incretin group and with little overlap of age between the DM groups. Likewise, it is true that 50% of all DM subjects were male, as were 50% of control subjects, but again 75% were male in the DM-incretin group, and only 33% were male in the DM-other group. Thus, we find the statements about matching to be misleading.

Other findings also raise some questions about comparability of the groups. Because pancreas weight was not available in seven (50%) of control subjects and because the large increase in β-cell mass in the DM-incretin group was calculated by Butler et al.[10] as the β-cell area × the pancreas weight, it is important to carefully examine the impact of pancreas weight on the calculations. Could it be that the crucial finding is a decrease of pancreatic weight in the DM-other group, rather than an increase in the DM-incretin group? If the DM-other group has a substantial number of subjects with covert type 1 DM, that may be playing a role, as there is a well-known decrease in weight in type 1 DM.[15,16] If type 1 DM is present, that might also account for the decrease in β-cell area in the DM-other group compared with the control group. And, the accuracy of the control group β-cell mass might be questioned in that four of the control subjects with the highest percentage of β-cell area lacked pancreas weight, by which β-cell mass is calculated. Thus, at the very least, there is an incomplete dataset for control subjects.

Another potentially important difference between the two DM groups is that in the nPOD Web site tabulation, amyloid was noted in nine (75%) of the DM-other subjects but only two (25%) of the DM-incretin subjects. Because islet amyloid may reflect β-cell apoptosis, this could contribute to the differences in β-cell area and mass reported. The presence of amyloid in a higher proportion of the DM-other subjects may be indicative that this group has more apoptosis and/or that the DM-incretin group has less amyloid because incretins are diminishing apoptosis.

In Butler et al.,[10] the authors noted "glucagon immunoreactive cells were frequently found in long linear groups or solid nests of cells either within the duct itself or in the immediate periductal location." However, they either did not detect or did not note that such staining could also be found in at least one control subject without DM (subject #6020), as shown in Figure 1.

The authors also claimed that there is an increase in pancreatic intraepithelial neoplasia (PanIN) cells in the DM-incretin group but failed to note that previous studies have shown an age-related increase in PanIN cells.[17] The nearly 20-year difference in age between the two DM groups could completely account for any difference in PanIN frequency. Butler et al.[10] asserted that GLP-1 receptors may be responsible for increase in exocrine pancreas and in PanIN cells but

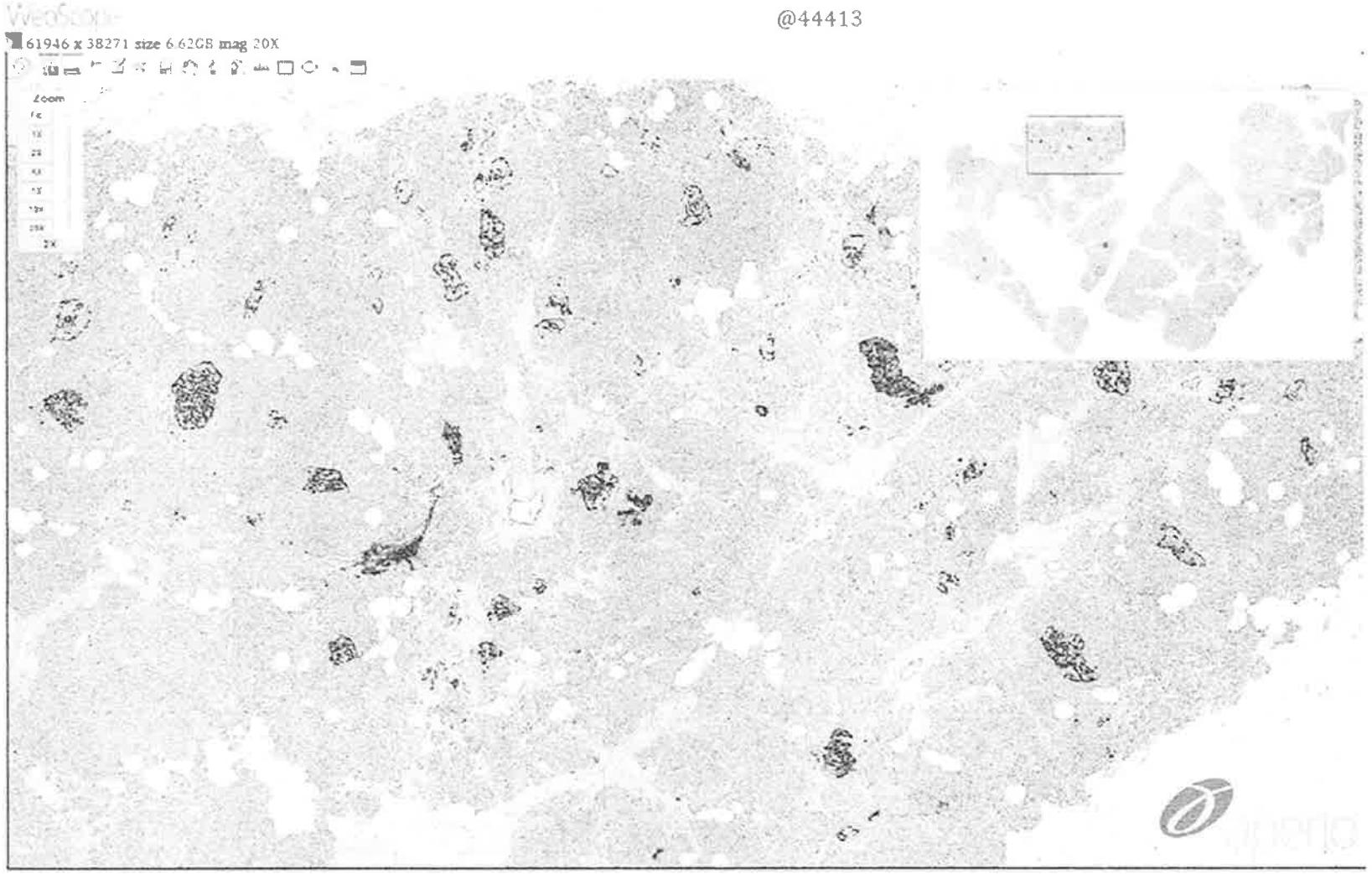

FIG. 1. A section from a control subject without diabetes shows staining for glucagon, including evidence of glucagon cells within pancreatic ducts. This figure was downloaded and enlarged from the Network for Pancreatic Organ Donors with Diabetes Web site at http://path-aperio.ahc.ufl.edu

failed to note that there is controversy as to whether GLP-1 receptors are expressed in such tissues, depending on which antibody is used to measure GLP-1 receptors, with multiple GLP-1 receptor antibodies, including several used to localize GLP-1 receptor expression in the pancreas, failing to exhibit appropriate sensitivity or specificity.[18]

Another point to note is that the authors argued that there is "α-cell hyperplasia, abnormal α-cell distribution and predisposition to glucagon expressing neuroendocrine tumors previously reported with suppressed glucagon secretion or signaling." However, the three articles cited include one in receptor knockout mice, one in receptor-deficient mice, and one in an individual with a mutation in the glucagon receptor.[19–21] No example is cited of such abnormalities in the setting of suppressed glucagon secretion, a known biological effect of GLP-1.

Butler et al.[10] also argued that β-cell function has not been shown to be improved on incretin therapies and used the continued presence of diabetes a year after treatment has commenced to conclude that the therapies have no effect. In fact, β-cell function has been carefully measured during therapy with incretins—both GLP-1 receptor agonists and dipeptidyl peptidase-4 inhibitors—and has been found to be increased.[22–24] It is true that diabetes has not been reversed, but improved glycemic control is maintained.

This critique describes our review of the data available on the nPOD Web site and our reading of the article by Butler et al.[10] From this, we conclude that the data and the implications of the data, as expressed by Butler et al.,[10] are vastly overstated and seemingly irresponsibly articulated. Their analysis seems to be more of an alarmist perspective, creating controversy rather than a neutral and fact-based approach. At the core of the discussion are limited numbers and many confounders related to subject history, presentation, and other subject-specific factors that make their conclusions invalid. We believe that a much larger sample needs to be examined, with appropriately matched subjects, including matching of DM subjects treated with incretins in comparison with DM subjects treated with other agents. Until such is accomplished, no conclusions can be made. In the interim, we note that the American Diabetes Association has launched a complete review of all industry data pertaining to the subject.[25] We also note that while our present critique was under review, the European Medicines Agency issued a statement that it had concluded its review prompted by the article of Butler et al.[10] and indicated "no new concerns for GLP-1 therapies identified on the basis of available evidence."[26]

A fundamental premise of all medical interventions is the calculation of benefit versus risk. In the case of the report by Butler et al.,[10] the beneficial clinical effects of the incretin drugs were ignored. Every drug should have aggressive pharmacovigilance to understand the full effects in heterogeneous populations. That often requires extensive experience with the drugs after approval by regulatory agencies. The irresponsible indictment of two classes of drugs that are used by millions of people, in our opinion, is reprehensible.

Acknowledgments

This research was performed by use of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative type 1 diabetes research project sponsored by JDRF. Figure 1 in this manuscript was provided by the nPOD online pathology site. Organ Procurement Organizations partnering with nPOD to provide research resources are listed at www.jdrfnpod.org/our-partners.php.

Author Disclosure Statement

E.H. declares no competing financial interests exist. J.L. reports serving on the Board of Directors of Dexcom and Steracycle and serving as a consultant to Anthelio Health Care and Serco UK. J.S.S. reports having served on the Board of Directors of Amylin Pharmaceuticals until the company was sold in August 2012 and is currently on the Board of Directors of Dexcom, Moerae Matrix, Paean Therapeutics, and VasoPrep Surgical, has consulted with BD Technologies, Bristol-Myers Squibb/Astra-Zeneca, Cebix, DiaVacs, Exsulin, Gilead, Halozyme, Ideal Life, Intarcia, MannKind, Mellitech, Merck, Orgenesis, Sanofi, Sekris, Takeda, Valeritas, and Viacyte, has had research grants (to the University of Miami) from Halozyme, Intuity Medical, Mesoblast, and Osiris Therapeutics, and currently holds stock in Dexcom, Ideal Life, Moerae Matrix, Patton Medical Devices, Tandem Diabetes Care, and VasoPrep Surgical.

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EXHIBIT 26

Exenatide Does Not Cause Pancreatic Tumors or Malignancies in Rats and Mice Following a 2-Year Period of Exposure

Abstract

Introduction

Methods

Table 1. Group distribution and doses of exenatide administration in mice

Table 2. Group distribution and doses of exenatide administration in rats

Table 3. Statistical comparison

Results

In-Life Observations

Figure 1. Survival during the 2-year carcinogenicity study

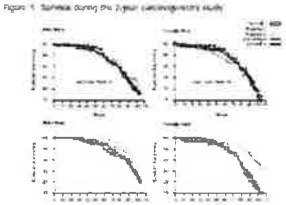


Figure 2. Food consumption values during the 2-year carcinogenicity study

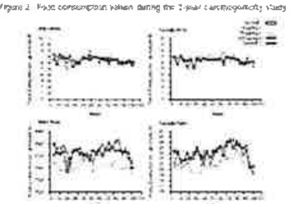
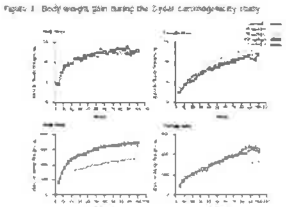


Figure 3. Body weight gain during the 2-year carcinogenicity study



Statistical Comparison

Microscopic Evaluation

Table 4. Summary of all primary proliferative lesions in the pancreas of mice and rats in the 2-year carcinogenicity studies

Discussion

References

Exhibit 26 - 303

EXHIBIT 27

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Nonclinical Evaluation of Pancreatic Safety with GLP-1 Based Therapies

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Pancreatitis, Diabetes, Pancreatic Cancer Workshop
National Institute of Diabetes and Digestive and Kidney Diseases, NIH
Bethesda, Maryland
June 12 – 13, 2013

Disclaimer: This presentation does not necessarily represent or convey changes in FDA guidance or policy

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Nonclinical Activities to Address Clinical Safety Signal

- Re-evaluated the nonclinical data from Investigational New Drug (IND) applications and New Drug Applications (NDA)
- Required the conduct of a pancreatic safety toxicology study in a rodent diabetes model
- Requested FDA's Division of Drug Safety Research to conduct studies in rodent disease models

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Toxicology Study Description

- Conducted with 2 species: rodent and nonrodent
- Often conducted at independent contract research labs
- Conducted per Good Laboratory Practice regulations (21 CFR 58)
- Study designs include an untreated control group and 3 treatment groups with 10 to 20 rodents/sex/group and 4 to 5 nonrodents/sex/group
- Dose levels range from clinically relevant doses to doses that provide 10- to 100-fold higher exposures than clinical doses
- Endpoints include: clinical signs, body weight, food consumption, hematology, coagulation, clinical chemistry, urinalysis, ECG measurements (nonrodents), organ weights, gross pathology, and histopathology for a battery of tissues including pancreas
- Additional endpoints can be added as needed

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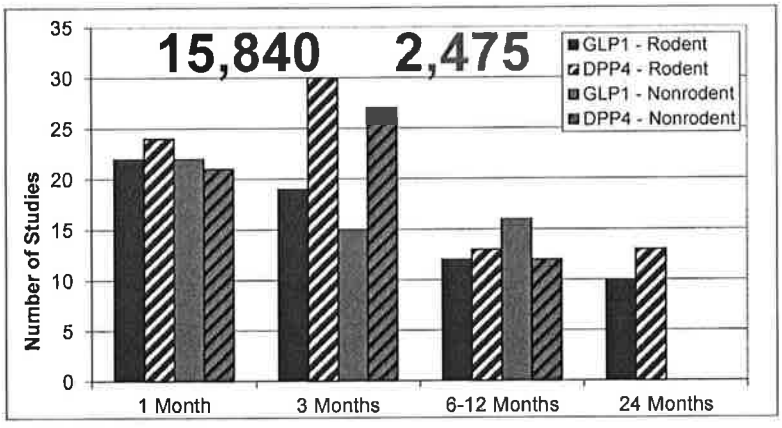
Number of Toxicology Studies Conducted with GLP-1 Based Therapeutics



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Pancreatic Findings in Healthy Animals

- Overt pancreatic toxicity, pancreatitis, or pancreatic tumors were NOT observed in standard toxicology studies conducted with healthy animals
- Treatment with GLP-1 based therapeutics occasionally resulted in small increases in incidence/severity of background lesions in some studies:
  - Amyloidosis
  - Islet cell hyperplasia/hypertrophy
  - Fibrosis
  - Lymphocytic infiltration/inflammation
  - Acinar cell atrophy
  - Acinar cell hyperplasia, focal
  - Ductal cell hyperplasia
- Unique treatment-related microscopic lesions were not observed

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Are There Predisposing Risk Factors?

- Diabetics often have one or more risk factors for pancreatitis
- Could the clinical signal be a disease-specific effect, due to other risk factors, or a human-specific effect?

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Studies in Disease Models

3-month pancreatic toxicity studies in Zucker diabetic fatty (ZDF) rats

- Conducted by Sponsors as Post-Marketing Requirements
- Protocols reviewed by FDA prior to study initiation
- The high doses were 10X to >50X of clinical exposure
- Extensive histopathology of the endocrine and exocrine pancreas and special staining to assess for increased proliferation, apoptosis, and duct morphology
- Results of 3 studies have been submitted to FDA
  - Liraglutide (Vrang et al., 2012)
  - Exenatide (Tatarkiewicz et al., 2013)
  - Sitagliptin (Forest et al., 2013; NIDDK meeting abstract)

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Studies in Disease Models - Results

- Data from 3 studies in ZDF rats (153 exposed to drug) did not show adverse treatment-related effects in the pancreas
- For one of these studies, FDA pathologists peer reviewed the pancreatic histopathology slides in a blinded manner (120 slides)
  - The opinions of the FDA pathologists largely agreed with the sponsor's report with regard to both type and severity of the observed changes
  - There were some differences in opinion with regard to the significance of the changes and the causative role of the drug
  - It was concluded that there was insufficient evidence to implicate the drug in off-target and mild inflammatory changes

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Studies Conducted by FDA

The Division of Metabolism and Endocrinology Products requested the Division of Drug Safety Research to conduct independent studies to evaluate the potential effects of GLP-1 based therapies on the pancreas

Objectives

- Confirm findings reported in literature
- Identify a model for improved screening of GLP-1 based therapeutics in development

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Models Evaluated by FDA

- Chemically-induced pancreatitis in mice
- ZDF and non-diabetic Sprague-Dawley rats
- C57BL6 mice fed a normal or high-fat diet
- Treatment included metformin, sitagliptin, and/or exenatide for 3, 6, or 12 weeks

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Results from FDA Studies

- Data from the pancreatitis and diabetic rat models did not confirm a treatment-related pancreatic signal
- High-fat fed C57BL6 mice
  - Some apparent treatment-related changes to acinar cells were detected in mice on HF diet after 12 weeks of treatment with exenatide.
  - The utility of the HF diet mouse model as a regulatory tool for evaluating the potency of compounds to induce pancreatic injury needs further evaluation.

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Summary

- Nonclinical programs for approximately 50 GLP-1 receptor agonists and DPP-4 inhibitors have not shown definitive treatment-related adverse effects in the pancreas
- Treatment with GLP-1 based therapeutics in a diabetes rodent model did not result in definitive adverse treatment-related effects in the pancreas
- Additional studies are warranted to identify a nonclinical model *(in vitro or in vivo)* that has regulatory utility *(e.g., widely available and reproducible)* in screening for potential pancreatic toxicity from current GLP-1 based therapeutics in development

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EXHIBIT 28

Review

Pancreatology

Pancreatology 2008;8:110–125
DOI: 10.1159/000123838

Published online: April 1, 2008

Pancreatic Carcinogenesis

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Key Words
Pancreatic cancer · Precursor lesion · Early detection · Mouse models · Genetics

Abstract
Pancreatic cancer is an almost universally lethal disease. Research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and acquired somatic mutations in cancer-associated genes. Multiple alterations in genes that are important in pancreatic cancer progression have been identified, including tumor suppressor genes, oncogenes, and genome maintenance genes. Furthermore, the identification of non-invasive precursor lesions of pancreatic adenocarcinoma has led to the formulation of a multi-step progression model of pancreatic cancer and the subsequent identification of early and late genetic alterations culminating in invasive cancer. In addition, an increased understanding of the molecular basis of the disease has facilitated the identification of new drug targets enabling rational drug design. The elucidation of genetic alterations in combination with the development of high-throughput sensitive techniques should lead to the discovery of effective biomarkers for early detection of this malignancy. This review focuses mainly on the current knowledge about the molecular insights of the pathogenesis of pancreatic ductal adenocarcinoma.

Epidemiology

Pancreatic cancer is a disease with a dismal outlook. In the United States approximately 33,000 patients are diagnosed with pancreatic cancer annually, and nearly an equal number will die from the disease, representing the fourth most common cause of cancer-related mortality. Men and women have an approximately equal risk [1]. Worldwide, pancreatic cancer causes an estimated 213,000 deaths each year [2]. For all stages combined, the 1-year survival rate is around 20%, and the overall 5-year survival rate is less than 5%, despite even the most aggressive therapies currently available [1].

A number of risk factors have been identified [3]. Pancreatic cancer is predominantly a disease of the elderly. Pancreatic cancer is rare before the age of 40, and the median age at diagnosis is 73 years. Cigarette smoking is by far the leading preventable cause of pancreatic cancer [4]. Cigarette smoking doubles the risk of pancreatic cancer (relative risk = 2) [3]. Other risk factors include diets high in meats and fat, low serum folate levels, obesity, long-standing diabetes mellitus, and chronic pancreatitis [3, 5–7]. Approximately 10% of patients demonstrate

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a familial predisposition for pancreatic cancer, and a subset of these patients harbor germline mutations in *BRCA2, p16/CDKN2A, PRSS1, STK11/LKB1*, or the DNA mismatch repair genes (see further discussion below). In the vast majority of patients with familial risk, however, the underlying genetic predisposition remains unknown.

Complete surgical resection remains the only curative treatment. Studies from high-volume centers with optimal staging report up to a 15–20% 5-year survival rate in patients undergoing surgical resection [8, 9]. The mortality rate is so high because pancreatic cancer usually only produces symptoms when it has already metastasized, and because there are no sensitive and specific tools to detect the disease at an earlier stage. Although multiple histological subtypes of pancreatic cancer have been described, the most common and deadliest form is pancreatic ductal adenocarcinoma [10]. Novel approaches to the management of patients with this aggressive disease are urgently needed.

Research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and acquired somatic mutations in cancer-associated genes. A compendium of alterations in tumor suppressor genes, oncogenes, and genome-maintenance genes that are important in pancreatic cancer progression have now been identified (fig. 1). This review focuses mainly on the molecular insights on pancreatic ductal adenocarcinoma and its precursor lesions, including insights gained through experimental models of pancreatic carcinogenesis.

Precursor Lesions of Pancreatic Cancer

Prior to a discussion on molecular genetics of pancreatic cancer, we will briefly discuss the current state of knowledge on precursor lesions of the pancreas. This is essential from the context of separating 'early' genetic changes (i.e. those associated with tumor initiation) from 'late' abnormalities (i.e. those associated with tumor progression). A recent review in *Pancreatology* has extensively discussed the histology and genetics of pancreatic cancer precursors [11]; therefore, we will only discuss these in fleeting detail. Briefly, pancreatic intraepithelial neoplasias (PanINs) are classified into a four tier classification, including PanIN-1A, -1B, -2, -3, reflecting a progressive increase in histologic grade culminating in invasive neoplasia (fig. 2). The lowest grade PanIN lesions can be flat (1A) or papillary (1B), but are characterized by absence of nuclear atypia and retained nuclear polarity. PanIN-2 lesions have micropapillary features with evidence of nuclear atypia and infrequent mitoses, while PanIN-3 lesions (a.k.a. carcinoma in situ) demonstrate widespread loss of polarity, nuclear atypia, and frequent mitoses. In addition to microscopic PanIN lesions, there are now recognized macroscopic (cystic) precursor lesions of pancreatic adenocarcinoma – including intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms. Akin to PanINs, the cystic precursor lesions also demonstrate a multistep histological and genetic progression to invasive neoplasia. Since IPMNs and mucinous cystic neoplasms can be detected by radiologic scans, they represent an opportunity to diagnose invasive pancreatic cancer before it can develop [11].

Tumor Suppressor Genes

Tumor suppressor genes are genes that promote tumor growth when inactivated. Tumor suppressor genes are recessive, i.e. the two copies need to be mutated for loss of function, and they can be inactivated by a variety of mechanisms. First, by an intragenic mutation in one allele (copy of a gene) coupled with loss of the second allele; second, through a deletion of both alleles (homozygous deletion), and third, by hypermethylation of the promoter of the gene-silencing gene expression. In sporadic cancers these alterations are both somatic mutations acquired during life, while patients with inherited forms of cancer inherit one mutant allele in the germline while the second allele is somatically mutated in the cancer cells.

The *p16INK4A/CDKN2A* gene, located on the short arm of chromosome 9 (9p), is one of the most frequently inactivated tumor suppressor genes in pancreatic cancer [12] (table 1). Remarkably, virtually all pancreatic carcinomas have loss of *p16INK4A/CDKN2A* function, in 40% of pancreatic cancer through homozygous deletion, in 40% by an intragenic mutation coupled with loss of the second allele, and in 15% by hypermethylation of the *p16INK4A/CDKN2A* gene promoter [12, 13]. The protein p16 belongs to the cyclin-dependent kinase (CDK) inhibitor family and functions to prevent the phosphorylation of Rb-1 by CDKs, and cyclin D-Cdk4 and cyclin D-Cdk6 complexes, which act as cell-cycle regulators [14, 15]. Loss of *p16INK4A/CDKN2A* results in inappropriate phosphorylation of Rb-1, thereby facilitating progression of the cell cycle through the G1/S transition [16].

Thus, the p16/Rb pathway is inactivated in virtually all pancreatic cancers, leading to an inappropriate progression through the G1 phase of the cell cycle. Of note, in a small group of patients, inherited mutations of the *p16INK4A/CDKN2A* gene cause the familial atypical multiple mole melanoma (FAMM) syndrome, which is associated with an increased risk of developing melanoma and an increased risk of developing pancreatic cancer [17, 18]. Particularly, the *p16* Leiden deletion, a 19-bp deletion, is associated with an increased pancreatic cancer risk [19].

In addition, the homozygous deletions, which inactivate *p16*, can encompass adjacent genes, including the *MTAP, IFNA1* and *IFNB1* genes [20, 21]. The *MTAP* gene is located approximately 100 kilobases telomeric to the *p16INK4A/CDKN2A* gene on chromosome 9p21, and is frequently contained in the *p16INK4A/CDKN2A* homozygous deletions. As a result, *MTAP* function is completely lost in approximately 30% of pancreatic adenocarcinomas. This is a potentially promising finding, because it may have therapeutic implications [22]. The product of the *MTAP* gene, the enzyme methylthioadenosine phosphorylase plays an important role in the synthesis of adenosine [23]. Chemotherapeutic agents, such as L-alanosine, a purine biosynthesis inhibitor, have been developed, to specifically target the selective loss of *MTAP* function in cancers, implicating that it might be effective against one third of the adenocarcinomas of the pancreas [22, 23].

Mutation of the *p53* gene on chromosome 17p is the most common somatic alteration in human cancer. The *p53* protein plays a central role in modulating cellular responses to cytotoxic stress by contributing to both cell-cycle arrest and programmed cell death. Loss of *p53* function during carcinogenesis can lead to inappropriate cell growth, increased cell survival, and genetic instability [24]. In pancreatic cancer, the *p53* tumor suppressor gene is inactivated in 50–75% of the cases and occurs predominantly through single allelic loss coupled with an intragenic mutation of the second allele [25]. The loss of *p53* means that two critical controls of cell number (cell division and cell death) are deregulated in the majority of pancreatic cancers. Of interest, *14-3-3σ*, a *p53*-regulated gene, plays a role in signal transduction, apoptosis, stress response and cytoskeletal organization [26]. *14-3-3σ* is transcribed in response to DNA damage and in a number of cancers it is an important mediator of *p53*-induced G2 arrest [27].

In addition, p53-induced growth arrest is also achieved by transactivation of *p21*. p53 binding to DNA stimulates production of the protein *p21*, which negatively regulates the complex consisting of cyclin D and the cell division-stimulating protein cyclin-dependent-kinase-2 [28], thereby preventing the cell from progressing from G1-S phase. This mechanism allows time for repair to damaged DNA. If *p53* mutated, it is not able to bind DNA, so *p21* is not made available and abnormal growth can occur. Cell lines which lack wild-type *p53* show a reduced or complete absence of *p21* [29]. Loss of *p21* activity has been observed in approximately 30–60% of pancreatic tumor specimens [30–32]. Pancreatic cell lines and pancreatic tumors show a correlation between active *p53* and *p21* [33].

As stated, *p53* loss is a 'double threat', because it results in both loss of cell cycle checkpoints, as well as deregulation of programmed cell death (i.e. apoptosis). It is now known that p53-induced apoptosis is mediated by activation of genes involved in the apoptotic pathway, for example genes such as *PUMA* (*p53*-upregulated modulator of apoptosis) and *Noxa*. *PUMA* and *Noxa* are activated in a *p53*-dependent manner following DNA damage. Once activated, they bind to Bcl-2, localize to the mitochondria to induce cytochrome c release, and activate the induction of programmed cell death [34–36].

Finally, the micro-RNA miR-34a deserves mention (miRNAs in general are discussed later): miR-34a is a direct transcriptional target of *p53*. MiR-34a activation can recapitulate elements of *p53* activity, including induction of cell-cycle arrest and promotion of apoptosis, and loss of miR-34a can impair *p53*-mediated cell death [37, 38]. Chang et al. [39] showed that reduced expression of miR-34a is a very frequent feature of pancreatic cancer cells.

DPC4 (Smad4) is a tumor suppressor gene on chromosome 18q and is one of the most commonly inactivated genes in pancreatic ductal adenocarcinoma, detected in approximately 55% of the cases. Inactivation occurs either through homozygous deletion, in approximately 30%, or loss of one allele coupled with an intragenic mutation in the second allele in approximately 25% [40–42]. The transcription factor SMAD4 is an important regulator of the transforming growth factor-β (TGF-β) signaling pathway [43]. Upon receptor activation, SMAD proteins become phosphorylated and heterodimerize with Smad4 to transmit upstream signals to the nucleus and transactivate transcription of specific target genes [44]. Loss of *SMAD4/DPC4* interferes with the intracellular signaling cascades downstream from TGF-β and activin, resulting in decreased growth inhibition via loss of pro-apoptotic signaling or inappropriate G1/S transition [43,

45]. The *SMAD4* gene is remarkable for two reasons. First, inactivation of the *DPC4* gene is relatively specific to pancreatic cancer, although it occurs with low incidence in other cancers, such as colon, breast, and ovarian or biliary tract carcinomas [46, 47]. Secondly, immunohistochemical labeling for Smad4 protein expression mirrors *DPC4/SMAD4* gene status in pancreatic cancers with rare exceptions [42]. Inactivation of *DPC4/SMAD4* is uncommon in nonductal neoplasms of the pancreas [10], and is rare in most extrapancreatic malignancies [10, 46]. Therefore, immunolabeling for loss of Smad4 is a convenient ancillary diagnostic marker in clinical specimens, including suspected metastases from an occult pancreatic primary.

Many other tumor suppressor genes that are targeted at low frequency in pancreatic cancer (<10%) deserve mentioning. Mutations in the *LKB1/STK11* gene are the cause of the autosomal-dominant inherited Peutz-Jeghers syndrome. Patients with Peutz-Jeghers syndrome have an increased risk of pancreatic cancer and it is conceivable that *LKB1* acts as tumor suppressor gene in pancreatic cancer as well [48, 49]. Intragenic mutations and homozygous deletions of the *MKK4* gene occur in a small percentage of pancreatic cancers [50]. The *MKK4* gene encodes for a component of a stress-activated protein kinase cascade and has a function in apoptosis and growth control. Furthermore, *MKK4* is preferentially inactivated in subsets of pancreatic cancer metastases, suggesting that the protein product may function as a metastasis suppressor [51]. Other less frequently affected tumor suppressor genes include the *TGF-β/activin* signaling pathway receptors such as the TGF-β type I receptor (*TGF-βR1; ALK5;* chromosome 9q), *TGFβR2* (chromosome 3p), *ACVR1β* (*ALK4;* chromosome 12q) [52] and *ACVR2* (chromosome 2q) [53, 54]. The TGFβR1 ALK5 forms a heterodimer with the TGF-β type II receptor (TGFβR2) to mediate signaling of *TGF-β* ligands. A downstream component of this pathway includes *DPC4 (SMAD4).* Signaling initiated after binding of *TGF-β*-related ligands to their cognate receptors leads to heteromerization and nuclear translocation of the Smad proteins and the transcriptional activation of target genes [55, 56]. TGF-β is a pleiotropic factor that regulates cell proliferation, angiogenesis, metastasis, and immune suppression. The involvement of the TGF-β pathway has been established in cancers of many organs including the breast, lung, colon and pancreas. TGF-β signaling is frequently attenuated in pancreatic cancer because of alterations in the components of the pathway [57, 58].

Oncogenes

Oncogenes are genes that contribute to oncogenesis when mutationally activated. In contrast to tumor suppressor genes they act in a dominant fashion, i.e. mutation of one copy of the gene suffices for activation. Oncogenes can be activated through a variety of mechanisms, including point mutations within the gene and amplification of the gene itself. A growing number of oncogenes have been identified that are targeted in pancreatic cancer.

The most common activating point mutation involves the *KRAS2* oncogene, on chromosome *12p*, in over 90% of pancreatic ductal adenocarcinomas [59, 60] (table 1). This is the highest fraction of K-ras alteration found in any human tumor type. Frequent mutation sites involve codons 12, 13 and 61, but in pancreatic ductal cancers the majority occur in codon 12. The *KRAS* gene product mediates signals from growth factor receptors and other signal inputs. Mutation of *KRAS* results in a constitutive gain of function, because the RAS protein remains trapped in the activated state even in the absence of growth factor signals, which leads to proliferation, suppressed apoptosis and cell survival.

The RAS family proteins encode small GTP-binding cytoplasmic proteins [44]. The constitutively active RAS intrinsically binds to GTP and confers uncontrolled stimulatory signals to downstream cascades including Ras effectors. Activated *KRAS* engages multiple effector pathways, notably the RAF-mitogen-activated protein kinase, phosphoinositide-3-kinase (PI3K) and RalGDS pathways.

Mutant *KRAS* has been extensively investigated as a marker of pancreatic cancer because mutations are basically entirely limited to one codon, can be readily detected using molecular assays and are present in approximately 90% of pancreatic ductal adenocarcinomas. Unfortunately, *KRAS* mutations are not specific for invasive pancreatic cancer and they occur in patients with chronic pancreatitis, in individuals who smoke, and in situ neoplasias from patients without pancreatic cancer [61, 62].

The *BRAF* gene on chromosome 7q is a member of the RAS-RAF-MEK-ERK-MAP kinase pathway, and is mutated in one-third of the pancreatic cancers with wild-type (normal) *KRAS* [63]. *BRAF,* a serine/threonine kinase located immediately downstream in RAS signaling, is a frequent mutational target in several cell lines and nonpancreatic primary cancers including 66% of melanomas and 10% of colorectal carcinomas [64, 65]. Interestingly, *KRAS* and *BRAF* mutations are mutually exclu-

sive and tumors with mutant forms of one of these 2 genes invariably retain wild-type copies of the other. The requirement of oncogenic *KRAS* or *BRAF* pathway-related signal transduction appears to be critically important for most instances of pancreatic ductal carcinogenesis.

The PI3K-kinase-AKT pathway is a key effector of *RAS*-dependent transformation of many cell types and plays a role in cell survival, cell proliferation and other growth-related processes [66]. Activated PI3K results in phosphorylated phosphatidylinositides (PIP3), a step inhibited by product of the tumor suppressor gene, *PTEN*. PIP3 in turn phosphorylates and activates AKT [29]. Recently activating mutations of *PIK3CA*, the gene encoding PI3K, have been reported in a subset of pancreatic cancer precursors, specifically in IPMNs [67]. Even in the absence of mutations, the PI3K/AKT pathway is constitutively active in the majority of pancreatic cancers [68]. This might be due to the aberrant expression of their natural antagonist *PTEN* [69]. Although *PTEN* is not mutated in pancreatic cancers, the reduction of its expression may give pancreatic cancer cells an additional growth advantage [70]. Furthermore, amplification or activation of *AKT2* kinase, a major target of the PI3K complex, occurs in up to 60% of pancreatic cancers [71–74], supporting the participation of an activated PI3K-AKT axis in this disease.

A third downstream pathway activated trough *RAS* is the RalGDS pathway. RalGDS is one of several known Ras-regulated guanine-nucleotide exchange factors, or *GEFs*, that function by activating Ral A and Ral B GTPases [75]. Recently, RAL A was shown to be activated in a variety of pancreatic cancers, and knockdown of *RAL A* suppressed tumorigenicity of RAS-transformed human cells [76]. In the same studies, knockdown of *RAL B* had no effect on tumor initiation, but suppressed tumor progression (i.e. metastases), suggesting divergent roles for the two RAL proteins in the context of pancreatic neoplasia. Whether or not these signaling moieties can be utilized as therapeutic targets remains to be determined.

The mammalian Hedgehog family of secreted signaling proteins – comprised of Sonic, Indian, and Desert Hedgehog (Shh, Ihh, and Dhh) – regulates the growth and patterning of many organs, including the pancreas, during embryogenesis [77]. The Hedgehog pathway is under negative regulation by the Patched (PTC) tumor suppressor protein that inactivates the Smoothed (SMO) protein. The Hedgehog ligands engage the PTC transmembrane protein, disrupting the inhibition of SMO and thereby enabling signaling transduction to the *GLI* family of transcriptional regulators [78]. Loss of *PTC*, activating mutations in *SMO* and overexpression of GLI and Hedgehog proteins are associated with a variety of cancers [79]. Activation of the Hedgehog pathway has been implicated in both the initiation of pancreatic ductal neoplasia and in the maintenance of advanced cancers [80]. The expression of the Hedgehog ligands, the transcriptional target gene *PTC*, and the essential pathway component *SMO* is undetectable in normal human pancreatic ducts. In contrast, a relative increase in the expression of these proteins is observed during pancreatic ductal tumorigenesis [78, 81, 82]. Moreover, it has been confirmed that the Hedgehog pathway plays a role in metastases. Inhibition of Hedgehog signaling has been shown to reduce the incidence of systemic metastasis in pancreatic adenocarcinoma xenografts [83]. Recently, Ji et al. [84] showed that there is a cross-talk between oncogenic *KRAS* and the Hedgehog signaling pathway in pancreatic cancer cell lines. Their studies suggest that oncogenic *KRAS* through the *RAF/MEK/MAPK* pathway suppresses GLI1 protein degradation and consequently plays an important role in activating Hedgehog signaling pathway in the absence of additional Hedgehog ligand during pancreatic tumorigenesis.

The Notch signaling pathway is another pathway which is important in directing cell fate and cell proliferation during embryonic development. Later in life, the Notch signaling pathway plays a critical role in maintaining the balance among cell proliferation, differentiation, and apoptosis [85]. In mammals, this signaling pathway involves interaction of the membrane-bound Notch receptors (Notch 1–4) and Notch ligands (Delta-like, and Jagged) on adjacent cells [85, 86]. The function of Notch signaling in tumorigenesis can be either oncogenic or antiproliferative, and the function is context dependent. In a limited number of tumor types, including human hepatocellular carcinoma and small cell lung cancer, Notch signaling is antiproliferative rather than oncogenic. However, most of the studies show an opposite effect of Notch in many human cancers including pancreatic cancer [87]. In the normal adult pancreas, Notch and its ligands are expressed at low levels. Interestingly, aberrant expression of its ligands, expression of mutant Notch1 oncoprotein, and abnormal expression of transcription targets of Notch signaling can be observed in early stages of pancreatic tumorigenesis as well as in invasive pancreatic cancer [88].

Several other oncogenes that are targeted in pancreatic cancer by amplifications deserve mentioning. First, the *AKT2* gene on chromosome 19q is a downstream effector of the PI3K/AKT pathway, and is amplified in 10–

Table 1. Frequency of selected tumor suppressor genes, oncogenes and genome maintenance genes

Gene mutations	Incidence in pancreatic adenocarcinoma, %
p16	80–95
p53	50–75
DPC4	45–55
K-RAS	75–90
BRAF	5–10 (estimated)
hMLH1, hMSH2	4
BRCA2	7–10

15% of pancreatic cancers [73, 89]. *AKT2* can be activated by stimuli such as platelet-derived growth factor, basic fibroblast growth factor, and insulin through the *PI3K/* AKT pathway, suggesting this pathway's importance in this tumor type [72]. Secondly, the AIB1 gene on chromosome *20q* is amplified in approximately 60% of pancreatic cancers [90]. The nuclear receptor coactivator amplified in breast cancer 1 *(AIB1/SRC-3)* belongs to the p160/ steroid receptor coactivator family *(SRC)* [91]. *AIB1* amplification and/or overexpression is not only detected in hormone-sensitive tumors, such as breast, prostate and ovarian, but it is also found in nonsteroid-targeted tumors such as pancreatic cancer, colorectal carcinoma and hepatocellular carcinoma [92]. Thirdly, the *MYB* gene on chromosome *6q* is amplified in 10% of pancreatic carcinomas [93]. Abnormalities in the locus of the human *MYB* gene have been observed in several human cancers. In a majority of these tumors, these abnormalities seem to be accompanied by an amplification of the *MYB* gene followed by enhanced transcription [94].

Genome Maintenance Genes

Genome maintenance genes are those that function to identify and repair damage to DNA. When a genome maintenance gene is inactivated, DNA damage is not repaired efficiently and DNA mutations accumulate. If these mutations occur in cancer-associated genes they can contribute to tumorigenesis [90]. Although gross chromosomal abnormalities are frequent in pancreatic ductal adenocarcinomas, genetic instability also occurs through DNA mismatch repair defects [95]. The DNA mismatch repair genes *hMLH1* and *hMSH2* are examples of genome maintenance genes targeted in pancreatic cancer [96]. When one of these genes is inactivated, DNA changes occur leading to 'microsatellite instability' (MSI). MSI is associated with poor differentiation, lack of *KRAS2* and *p53* mutations, and germline mutations of this gene are associated with the human nonpolyposis colorectal cancer syndrome (HNPCC) [96–98]. Approximately 4% of pancreatic cancers have MSI and these cancers have a specific microscopic appearance called 'medullary type', which includes a syncytial growth pattern, pushing borders and lymphocytic infiltrate [96].

The causative genes of Fanconi anemia, *FANCC* and *FANCG*, also play a role in pancreatic tumorigenesis [99]. Fanconi anemia is a hereditary cancer susceptibility disorder, with the occurrence of hematologic abnormalities or acute myelogenous leukemia at an early stage, usually leading to death before the age of 20. Patients who survive into adulthood often develop solid tumors [99]. The *BRCA2* gene represents Fanconi complementation group D1 and is thought to aid DNA strand and interstrand crosslinking repair. *BRCA2* has therefore been categorized as genome maintenance gene rather than a standard tumor suppressor. In ductal pancreatic cancers 7–10% harbor an inactivating intragenic inherited mutation of one copy of the *BRCA2* gene, accompanied by loss of heterozygosity [100, 101]. Of interest, it has been shown that the presence of *BRCA2*/Fanconi anemia gene mutations in pancreatic cancer may make them particularly sensitive to chemotherapeutic agents that cause DNA crosslinks such as Mitomycin C, because these cancers are unable to repair DNA interstrand crosslinks [102].

Growth Factors

Several of the genes known to be overexpressed in pancreatic cancer include growth factors and their receptors. Growth factors are the proteins that control cell differentiation and proliferation. Disturbances in growth inhibition and an abundance of growth-promoting factors give cancer cells a distinct growth advantage, which clinically results in rapid tumor progression. The epidermal growth factor receptor (EGFR) is overexpressed and plays a distinct role in pancreatic cancer. The four receptors of the EGF family are membrane-spanning glycoproteins composed of an amino terminal extracellular ligand-binding domain, a hydrophobic transmembrane region and a cytoplasmic domain that contains both the tyrosine kinase domain as well as the receptor [103]. The classical EGF receptor is also known as HER1 or ErbB-1. The remaining three receptors are designated HER-2/ Neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4).

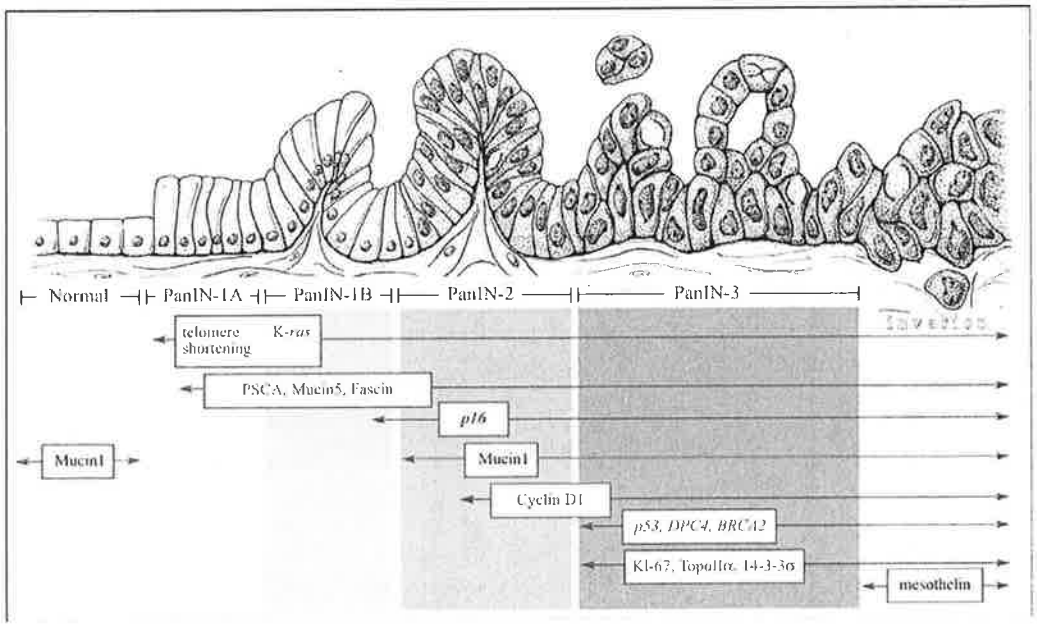

Fig. 1. Progression model of pancreatic ductal adenocarcinoma from normal (left) to carcinoma (right). The histological progression is associated with the accumulation of specific genetic alterations. Reprinted with permission from Maitra et al. [164].

HER-2/neu overexpression is most prominent in well-differentiated ductal adenocarcinoma, as well as in the early-stage precursor lesions, and appears to correlate with the grade of dysplasia in the precursor lesions [104, 105]. In pancreatic cancer, *HER-2/neu* amplification has been observed with a variable incidence of 10–60% [106, 107]. In addition, increased levels of fibroblast growth factor (FGF), FGF-receptor, insulin-like growth factor I (IGF-I), IGF-I receptor, nerve growth factor, and vascular endothelial growth factor (VEGF) are also reported in pancreatic cancer [108, 109].

Tumor growth requires accompanying expansion of the host vasculature with tumor progression, which is often correlated with vascular density. VEGF is the best-characterized inducer of tumor angiogenesis. Interestingly, Delta-like ligand 4 (Dll4), a Notch ligand, is dynamically regulated by VEGF [110]. Several studies demonstrated that Dll4 may act downstream of VEGF as a 'brake' on VEGF-mediated angiogenic sprouting [111]. Dll4, a transmembrane ligand for the Notch family of receptors, is induced by VEGF as a negative feedback regulator and acts to prevent overexuberant angiogenic sprouting [112].

Telomere Shortening

Defective telomeres may be the major cause of the chromosomal instability observed in many cancers and in the vast majority of pancreatic cancers [113]. Telomeres are structures at the end of linear chromosomes that normally function to protect the terminal sequences and prevent the ends of chromosomes from joining aberrantly [114, 115]. Telomeres serve as protective 'caps' and are composed of short repeated DNA sequences and associated proteins. It appears that telomeres become abnormally short very early in the development of pancreatic neoplasia [114]. These shortened telomeres can presumably lead to the abnormal fusion of chromosome ends and in this fashion to chromosome instability, promoting further neoplastic progression in these cells [90]. Such a chromosome fusion leads to so-called anaphase bridges during mitosis [116]. These anaphase bridges frequently break during cellular replication, generating unstable chromosome ends that are subject to abnormal fusion events and subsequent chromosomal rearrangements [117]. This process, called breakage-fusion-bridge cycles, has been observed in pancreatic cancers and is

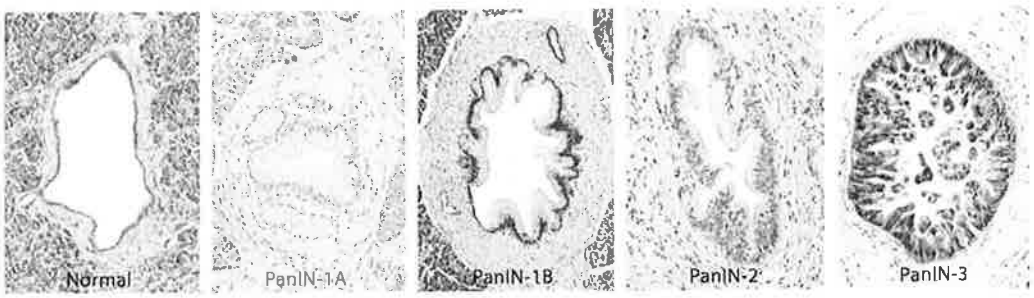

Fig. 2. Consecutive PanIN lesions with progressive histological changes from normal to PanIN-3. Reproduced with permission from http://pathology.jhu.edu/pancreas_panin.

believed to be one of the major causes underlying loss of function of tumor suppressor genes and the gain of function of oncogenes as described earlier [90]. In most instances, cells harboring this degree of genomic instability are eliminated through activation of *p53*. However, chromosomal rearrangements likely persist in cells with *p53* mutations, and these cells will then quickly accrue further genomic alterations [118]. Thus telomere dysfunction and *p53* loss cooperate to promote the development of carcinomas in multiple tissues [79]. Chromosomal instability provides a tumor with the genetic diversity to overcome certain barriers in carcinogenesis. However, ultimately, chromosomal instability might prove counterproductive to tumor growth, which may explain why neoplasms seem to acquire mechanisms to elongate their telomeres at later stages in the development of a malignancy, often through the reactivation of the enzyme telomerase, or through alternate lengthening of telomeres [119].

Familial Pancreatic Cancer

In the majority of cases, cancer is a multifactorial disorder in which genetic and environmental factors interact to initiate carcinogenesis. However, in a minority, the disease follows a familial pattern of transmission, suggesting a hereditary cancer syndrome. Characterization of the genetic mutations segregating in such families has helped to elucidate the molecular events that underlie tumorigenesis in the more common multifactorial form of the disease. Elucidation of the mechanisms of hereditary colorectal cancer and breast/ovarian cancer syndromes represents some of the greatest triumphs of the last century in the field of cancer genetics.

It has been estimated that 10% of pancreatic cancers have a familial basis [120, 121]. Having a first-degree relative with pancreatic cancer doubles the risk of developing pancreatic cancer [122], and the risk increases with increasing numbers of affected relatives [123]. Segregation analyses have suggested that an autosomal dominant pattern of inheritance is the most parsimonious genetic model for this increased risk [124], but the gene responsible for the familial aggregation of pancreatic cancer in the majority of cases has not yet been identified [125]. In different countries familial pancreatic cancer registries have been established to investigate the epidemiology and genetic background of these families, and to organize the screening programs for high-risk relatives and for follow-up. The largest such registry, the National Familial Pancreas Tumor Registry, is located at the Johns Hopkins Medical Institutions, Baltimore, Md., USA (http://pathology2.jhu.edu/pancreas/nfptr.cfm) [125].

To date, at least five hereditary disorders that significantly increase the risk of pancreatic cancer have been described. These include familial breast/ovarian cancer syndrome (caused by inherited mutations in the *BRCA2* gene), the FAMM syndrome (caused by germline mutations in the *p16* gene), the Peutz-Jeghers syndrome (caused by inherited mutations in the *STK11/LKB1* gene), hereditary pancreatitis (caused by germline mutations in the *PRSS1* gene), and hereditary HNPCC caused by mutations in *hMLH1* or *hMSH2*.

Familial breast/ovarian cancer syndrome is associated with an increased risk of breast cancer in men and women, and a subset of these families also harbor an increased risk for pancreatic cancer [126]. Germline mutations of the *BRCA2* gene, residing on 13q12–13, are identified in 4–17% of familial pancreatic cancer, with a particular propensity for occurring in families of Ashkenazi Jewish

heritage [100, 127]. As mentioned earlier, the protein product of the *BRCA2* gene has been shown to interact with protein products of several of the Fanconi anemia genes and to function in the repair of double-strand DNA breaks [99].

The FAMM syndrome is an autosomal dominant disorder characterized by the familial occurrence of multiple melanocytic naevi, atypical naevi, and an increased risk of both melanoma and pancreatic cancer [128, 129]. FAMM can be caused by germline mutations in the *p16/CDKN2A* gene on chromosome 9p. The carriers of the germline *p16*-Leiden mutation have an estimated risk of 17% to develop pancreatic cancer by the age of 75 years [19, 130].

The Peutz-Jeghers syndrome is a rare, autosomal dominant condition characterized by the development of hamartomatous gastrointestinal polyps, mucocutaneous pigmentation and high lifetime risk of developing cancer, affecting both gastrointestinal and extragastrointestinal sites. The lifetime risk of developing pancreatic cancer is approximately 36% [131]. In 50% of families the pathogenesis is caused by germline mutations occurring in the *STK11/LKB1* gene [48, 132].

Hereditary pancreatitis is characterized by the familial occurrence of pancreatitis with an early age of onset [133]. Germline mutations in the *PRSS1* gene cause an autosomal dominant form of the disease, whereas germline mutations in *SPINK1* lead to an autosomal recessive pattern of inheritance. An estimated 40% of patients with familial pancreatitis will develop pancreatic cancer by the age of 70 years [134].

HNPCC has an autosomal dominant pattern of inheritance, it affects approximately 1 in 200 persons and is associated with multiple forms of cancer, most importantly colorectal, but also gastric, endometrial, and pancreatic cancer [135]. As discussed before, HNPCC is caused by mutations in one of the DNA mismatch repair genes. The group of individuals with a known predisposing familial syndrome, and with a history of familial pancreatic cancer would be among the first to benefit from screening tests for early detection of pancreatic cancer.

Mouse Models of Pancreatic Cancer

Although the pancreas was the first organ where transgenesis was attempted over two decades ago [136], the development of a mouse model that faithfully recapitulates the multistep progression of human pancreatic adenocarcinoma has been elusive. In 2003, Hingorani et al. [137] developed a mouse model of pancreatic neoplasia by conditional mis-expression of mutant *KRAS* in the pancreas from its endogenous promoter. The bitransgenic mice express a 'knock-in' $Kras^{G12D}$ upon Cre-mediated recombination and removal of a lox-STOP-lox allele within the *Pdx1* expression domain. *Pdx1* is a transcription factor that is expressed in the developing pancreas and foregut, restricting mutant *KRAS* expression to these organs. The Pdx1-Cre, lox-STOP-lox-$Kras^{G12D}$ mice develop the entire histologic compendium of murine PanIN (mPanIN) lesions observed in the cognate human disease, and a subset of mice develop invasive pancreatic carcinomas as well. Subsequent models have utilized additional cooperating mutations with *Kras* (for example, an oncogenic $Trp53^{R172H}$ allele or biallelic deletions of INK4a/Arf) – these compound transgenic mice develop metastatic pancreatic cancers with near-universal penetrance, and represent biologically relevant models of advanced pancreatic cancer in humans [138–140].

Several important lessons have been learnt from these newly developed mouse models of pancreatic cancer. First, these studies indicate the likely absolute requirement of mutant *Kras* in order to initiate pancreatic neoplasia along the mPanIN pathway, which might also explain the extremely high frequency of *KRAS* abnormalities in human PanIN lesions and pancreatic cancer [141]. Thus, misexpression of other oncogenes by themselves results in pancreatic 'cancer' in mice (for example, aberrant expression of the Hedgehog transcription factor GLI2) [82], but it is only upon coexpression with mutant *Kras* that these mice develop cancers preceded by mPanINs. Second, the expression of mutant *Kras* from its endogenous promoter appears to be a prerequisite as well, since earlier models of transgenic *Kras* expression have resulted in cancers of acinar histogenesis without mPanIN formation [142]. Third, these mouse models have helped elucidate some insights into the putative cell-of-origin of pancreatic cancer. For example, recent studies by Guerra and colleagues have demonstrated that mPanINs and adenocarcinomas can be reproduced in the pancreas of adult mice by conditional misexpression of mutant *Kras* to the elastase-expressing acinar/centroacinar compartment [143]; the one caveat is that the mature acinar/centroacinar compartment appears to be resistant to the oncogenic transformation unless accompanied by an ongoing injurious stimulus (i.e. chronic pancreatitis). These studies provide remarkable experimental reiteration to the long-standing epidemiological associations between chronic pancreatitis and an increased incidence of pancreatic cancer [3]. They also underscore the possibility that the mon-

iker of 'ductal' adenocarcinoma might not reflect the true histogenesis of these cancers, at least in the context of murine pancreatic neoplasia. Fourth, and not the least, the development of these models have provided an unprecedented opportunity to explore preclinical diagnostic and therapeutic strategies in autochthonous models not afforded by short-term xenograft studies. For example, the cancers developing in these mice recapitulate not only the morphology of the cognate human disease, but also many of the oncogenic signaling pathways like *EGFR*, *Notch* and *Hedgehog* [137, 140]. Small molecule inhibitors targeted against these pathways can now be tested in the transgenic models prior to clinical trials. There is little doubt that the development of these models has fulfilled a critical lacuna on the field of pancreatic cancer research.

Molecular Biomarkers and Therapy

The gene expression patterns in pancreatic cancer have been studied using multiple platforms. A decade ago, gene expression was studied through analysis of the product of one gene at a time. Currently, gene expression patterns can be studied using technologies that assay nearly the entire genome simultaneously. Examples of such technologies that have been applied to pancreatic cancer include serial analysis of gene expression, cDNA arrays and oligonucleotide arrays [144–147]. The protein products of differentially expressed genes have proven useful as diagnostic markers in tissue biopsies, as serum markers, and as therapeutic targets. For example, prostate stem cell antigen and mesothelin were identified to be overexpressed in the majority of pancreatic cancers by serial analysis of gene expression, and immunolabeling for these two proteins can be used to aid in the interpretation of challenging pancreatic biopsies [148, 149]. Similarly, osteopontin was identified as overexpressed in pancreatic carcinoma using oligonucleotide microarrays, and serum osteopontin levels have a sensitivity of 80% and a specificity of 97% for pancreatic cancer [150].

Recently, micro-RNAs (miRNAs), a novel class of 18–23 nucleotide noncoding RNAs, have gained attention as another family of molecules involved in cancer development. Current evidence has illustrated that miRNAs are misexpressed in various human cancers, and further indicates that miRNAs can function as tumor suppressors ('TSGmiRs') or oncogenes ('oncomiRs') [151, 152]. Upon binding to their target RNAs, miRNAs cause posttranscriptional gene silencing by either cleaving the target mRNA or by inhibiting the translation process [153].

As several studies have highlighted, miRNA expression is deregulated in pancreatic cancer. A miRNA signature of pancreatic cancer has been elucidated, and it includes the upregulation of miR-21, miR-155, miR-221 and miR-222 [154, 155]. Moreover, Chang et al. [39] found that miR-34a is frequently lost in pancreatic cancer cell lines. These studies demonstrate that miRNAs may become useful biomarkers for pancreatic cancer diagnostics. In addition, these aberrantly expressed miRNAs might be useful as potential therapeutic targets, with the recent availability of in vivo miRNA knockdown strategies ('antagomirs') [156].

The revolution in our understanding of the genetics of cancer and the exploration of gene expression on a large scale has brought with it the hope that novel therapies can be developed specifically exploiting the genetic deletions and resultant absolute biochemical deficiencies present in pancreatic cancer. Two promising examples of therapies using a specific biochemical difference, including mitomycin C for pancreatic cancers harboring *BRCA2* gene mutations and L-alanosine, a purine biosynthesis inhibitor, for pancreatic cancers with loss of *MTAP* function were already mentioned above.

The downregulation of Notch signaling could also be a novel therapeutic approach for pancreatic cancer. Numerous studies have proposed inhibition of Notch signaling as a strategy for cancer treatment, such as with the pharmacological block of γ-secretase enzyme with small molecule inhibitors, which has a striking antineoplastic effect in Notch expressing transformed cells in vitro and in xenograft models [157]. Inhibitors of γ-secretase prevent the second ligand-induced proteolytic cleavage of the Notch receptor, thereby blocking the Notch signaling pathway. Importantly, in pancreatic cancer cells it has been shown that downregulation of Notch1 inhibits cell growth and induces apoptosis [87]. In other compartments of the gastrointestinal tract, notably the colorectum and the esophagus, regression of tumorigenesis is observed after chemical inhibition of Notch [158, 159].

Furthermore, developmental signaling pathways, like the Hedgehog signaling pathway, have emerged as therapeutic targets in pancreatic cancers [160]. This pathway is aberrantly activated in the majority of pancreatic ductal adenocarcinomas [78]. Drugs such as cyclopamine which specifically inhibit the Hedgehog pathway have been shown to be effective in xenograft models of human pancreatic cancer in treated mice [81]. Interestingly, the realization of cross-talk between *RAS/MAPK* and Hedgehog signaling pathways in pancreatic carcinomas also suggest that targeting the *RAS* and Hedgehog pathways

synergistically may represent a new therapeutic strategy [84]. Additionally, there are a few promising agents on the therapeutic horizon, being tested in clinical trials, like bevacizumab, the monoclonal antibody against VEGF, which targets tumor vascularization and cetuximab, the monoclonal antibody against the EGFR [161]. Of note, trastuzumab (Herceptin®) is a humanized monoclonal antibody that acts on the HER2/neu (erbB2) receptor, a member of the EGFR family, and shows profound beneficial results with breast cancer patients whose tumors overexpress this receptor [103]. Whether trastuzumab will be as effective a form of treatment in pancreatic cancer as it appears to be in breast cancer, is currently the focus of several studies [162, 163].

Future Perspectives

Intensive research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and/or acquired somatic mutations in cancer-associated genes. It has uncovered multiple alterations in many genes that are important in pancreatic cancer progression. In addition, an increased understanding of the molecular basis of the disease has provided the identification of new drug targets enabling rational drug design, and facilitated the production of animal models of the disease on which such therapies can be tested.

Pancreatic ductal adenocarcinoma is nevertheless still one of the most lethal cancers of all human malignancies. The poor prognosis and late presentation of pancreatic cancer patients emphasize the importance of early detection, which is the sine qua non for the fight against pancreatic cancer. It is hoped for the future that the understanding of genetic alterations in combination with the development of high-throughput sensitive techniques will lead to the rapid discovery of an effective biomarker.

Acknowledgements

This study was supported by the Sol Goldman Pancreatic Cancer Research Center, the Michael Rolfe Foundation for Pancreatic Cancer Research, NIH R01CA113669 and NIH P50CA062924.

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EXHIBIT 29

Design of the liraglutide effect and action in diabetes: Evaluation of cardiovascular outcome results (LEADER) trial

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Background Diabetes is a multisystem disorder associated with a nearly twofold excess risk for a broad range of adverse cardiovascular outcomes including coronary heart disease, stroke, and cardiovascular death. Liraglutide is a human glucagon-like peptide receptor analog approved for use in patients with type 2 diabetes mellitus (T2DM).

Study Design To formally assess the cardiovascular safety of liraglutide, the Liraglutide Effect and Action in Diabetes: Evaluation of cardiovascular outcome Results (LEADER) trial was commenced in 2010. LEADER is a phase 3B, multicenter, international, randomized, double-blind, placebo-controlled clinical trial with long-term follow-up. Patients with T2DM at high risk for cardiovascular disease (CVD) who were either drug naive or treated with oral antihyperglycemic agents or selected insulin regimens (human NPH, long-acting analog, or premixed) alone or in combination with oral antihyperglycemics were eligible for inclusion. Randomized patients are being followed for up to 5 years. The primary end point is the time from randomization to a composite outcome consisting of the first occurrence of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke.

Conclusions LEADER commenced in September 2010, and enrollment concluded in April 2012. There were 9,340 patients enrolled at 410 sites in 32 countries. The mean age of patients was 64.3 ± 7.2 years, 64.3% were men, and mean body mass index was 32.5 ± 6.3 kg/m^2. There were 7,592 (81.3%) patients with prior CVD and 1,748 (18.7%) who were high risk but without prior CVD. It is expected that LEADER will provide conclusive data regarding the cardiovascular safety of liraglutide relative to the current standard of usual care for a global population of patients with T2DM. (Am Heart J 2013;166:823-830.e5.)

Background

Diabetes mellitus affects nearly 350 million people worldwide,[1] including 26 million patients in the United States,[2] and the prevalence continues to increase. Diabetes is a chronic disease associated with long-term vascular complications. Type 2 diabetes mellitus (T2DM) is a multisystem disorder that is also independently associated with a nearly twofold excess risk for a broad range of adverse cardiovascular outcomes including coronary heart disease (CHD), stroke, and cardiovascular death. Subgroups of individuals with diabetes at lower absolute risk of cardiovascular complications, including women, younger persons, nonsmokers, and persons with below average blood pressure, also have an elevated risk of micro- and macrovascular complications (including CHD), compared with persons without diabetes.[3]

Effective strategies to mitigate cardiovascular risk and prevent or reduce the occurrence of microvascular complications are the cornerstone of treatment for patients with diabetes.[4] These measures include lifestyle management, smoking cessation, and individualized risk

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Submitted March 26, 2013; accepted July 3, 2013.

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0002-8703/$ - see front matter

http://dx.doi.org/10.1016/j.ahj.2013.07.012

factor treatment for low-density lipoprotein cholesterol and blood pressure. Glycemic control significantly reduces the development and progression of microvascular complications. Although metformin is the mainstay of initial therapy, treatment with a glucagon-like peptide-1 (GLP-1) receptor agonist is now regularly used as an add-on approach to achieve glycemic control.[5]

Although available diabetes therapies clearly improve glycemic control, the cardiovascular safety of particular glucose-lowering agents is controversial. When cardiovascular safety concerns were identified in an agonist of the peroxisome proliferator–activated receptor gamma class,[6] as well as in a separate federally funded study examining tight glycemic control in general,[7] the United States Food and Drug Administration subsequently issued mandatory guidelines to manufacturers for evaluating the cardiovascular safety of emerging therapies to treat diabetes.[8] Before new diabetes drug approval, manufacturers are now required to perform an integrated meta-analysis of completed studies to demonstrate an estimated relative risk with upper two-sided 95% confidence limits for major adverse cardiovascular events of <1.8 versus comparators. If the upper limit is 1.3 to 1.8, safety must subsequently be demonstrated in postmarketing cardiovascular outcome trials to rule out an upper confidence limit of 1.3.

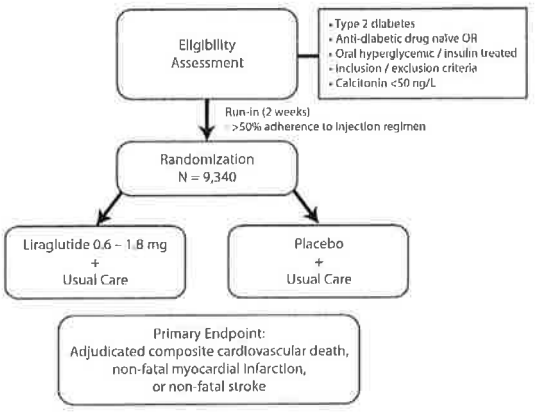
Glucagon-like peptide-1 and liraglutide. Native GLP-1 is an incretin hormone produced in the gut and secreted in response to food consumption.[9] The main pharmacological effects of GLP-1 are stimulation of endogenous insulin in response to elevated glucose, suppression of elevated glucagon, and regulation of satiety/appetite[10,11] and are all potentially beneficial or impaired in T2DM.

Liraglutide is an analog of human GLP-1 approved for use in patients with T2DM. Liraglutide has 97% homology with human GLP-1 and is administered subcutaneously once daily. Its glucose-lowering efficacy has been established, and its use results in hemoglobin A1c (HbA1c) reductions of 1.0 to 1.5% in addition to moderate weight loss across a wide range of patient types.[12] To formally assess the cardiovascular safety of liraglutide, the Liraglutide Effect and Action in Diabetes: Evaluation of cardiovascular outcome Results (LEADER) trial (clinicaltrials.gov NCT01179048) was commenced in 2010. This article reports the study design and baseline characteristics of the study population.

Study design

Objective. The primary objective of LEADER is to assess the effect of treatment with liraglutide compared to placebo (for at least 3.5 years and up to 5 years) on the incidence of cardiovascular events, as defined by the primary end point of cardiovascular death, nonfatal myocardial infarction (MI), or nonfatal stroke in adult patients with T2DM. The primary analysis will be noninferiority testing. If the prespecified noninferiority criteria are met, then superiority testing will be performed. Noninferiority of liraglutide versus placebo will be assessed by inspecting the upper range of the two-sided 95% CI, and noninferiority will be established if that upper range is <1.3. Only if noninferiority is established for the primary outcome will the data then be used to test for evidence of a lower outcome hazard with liraglutide over placebo. Superiority will be established if the hazard ratio of the upper range of the two-sided 95% CI is <1.0. This approaches a closed testing procedure, and therefore, no adjustment of the significance level is required.

Figure 1



LEADER study design.

Patient population. LEADER is a phase 3B, multicenter, international, randomized, double-blind, placebo-controlled clinical trial with long-term follow-up (Figure 1). Male and female patients with T2DM, who were either drug naive or treated with oral antihyperglycemic agents or selected insulin regimens (human NPH, long-acting analog, or premixed) alone or in combination with oral antihyperglycemics were eligible for inclusion.

LEADER enrolled 2 distinct populations of high-risk patients either with or without prior cardiovascular disease (CVD): (1) patients with prior CVD were ≥50 years old and had one or more of the following cardiovascular comorbidities (detailed criteria are shown in Table I): concomitant CVD, cerebrovascular disease, peripheral vascular disease, chronic renal failure, or chronic heart failure; (2) patients without prior CVD were ≥60 years old at screening and had one or more cardiovascular risk factors shown in Table I. Enrollment of approximately 400 patients with moderate (30-59 mL/min per 1.73 m^2) and 200 patients with severe (<30 mL/min per 1.73 m^2) reductions in baseline estimated glomerular filtration rate (eGFR) as estimated using the Modification of Diet in Renal Disease (MDRD)

Table I. LEADER Inclusions and Exclusions

Inclusion criteria
- Type 2 diabetes
- Anti-diabetic drug naïve or treated with one or more oral anti-diabetic drugs or treated with human NPH insulin or long-acting insulin analogue or premixed insulin, alone or in combination with OAD(s)
- HbA1c ≥7.0%
- Prior CVD cohort: age ≥50 and ≥1 of the following criteria.
  - Prior MI
  - Prior stroke or TIA
  - Prior coronary, carotid or peripheral arterial revascularization
  - >50% stenosis of coronary, carotid, or lower extremity arteries
  - History of symptomatic CHD documented by Positive exercise stress test or any cardiac imaging or Unstable angina with ECG changes
  - Asymptomatic cardiac ischemia
    Documented by positive nuclear imaging test, exercise test or dobutamine stress echo
  - Chronic heart failure NYHA class II-III
  - Chronic renal failure,
    eGFR <60 mL/min per 1.73m^2 MDRD
    eGFR <60 mL/min per Cockcroft-Gault formula
- No Prior CVD group: Age ≥60 y and ≥1 of the following criteria.
  - Microalbuminuria or proteinuria
  - Hypertension and left ventricular hypertrophy by ECG or imaging
  - Left ventricular systolic or diastolic dysfunction by imaging
  - Ankle-brachial index <0.9

Exclusion criteria
- Type 1 diabetes
- Calcitonin ≥50 ng/L
- Use of a GLP-1 receptor agonist (exenatide, liraglutide or other) or pramlintide or any DPP-4 inhibitor within the 3 months prior to screening
- Use of insulin other than human NPH insulin or long-acting insulin analogue or premixed insulin within 3 months prior to screening. Short-term use of other insulin during this period in connection with intercurrent illness is allowed, at Investigators discretion
- Acute decompensation of glycemic control
- An acute coronary or cerebrovascular event in the previous 14 d
- Currently planned coronary, carotid, or peripheral artery revascularization
- Chronic heart failure (NYHA class IV)
- Current continuous renal replacement therapy
- End-stage liver disease
- History of solid organ transplant or awaiting solid organ transplant
- Malignant neoplasm
- Family or personal history of multiple endocrine neoplasia type 2 (MEN2) or familial medullary thyroid carcinoma (FMTC)
- Personal history of non-familial medullary thyroid carcinoma

equation was also prespecified. Exclusion criteria are listed in Table I.

Treatment regimen. After eligibility assessment and informed consent, patients completed a minimum 2-week run-in period consisting of a daily single-blind subcutaneous injection of placebo. Patients demonstrating ≥50% adherence to the regimen and willingness to continue with the injection protocol throughout the trial then underwent randomization, which was carried out for all subjects using the interactive voice/web response system. Subjects meeting all inclusion/exclusion criteria were randomized in a 1:1 manner to receive a double-blind, once-daily maximum dose of liraglutide 1.8 mg or equivalent placebo as an add-on to their standard-of-care treatment. Liraglutide was administered at 0.6 mg daily for 1 week, 1.2 mg for an additional week, and a potential maximum dosage thereafter of 1.8 mg based on tolerance, as determined by the investigator.

Table II. Standard of Care Guidelines for LEADER

Blood glucose
- HbA1c ≤7.0% (individualized depending on patient).
- If >7.0%, additional HbA1c measurement after 3 m. If HbA1c still >7.0%, treatment should be intensified to achieve target if appropriate.

Therapy
- Lifestyle modifications and metformin are considered foundational therapy in most countries
- Intensification:
  - Add-on therapy: thiazolidinediones, sulfonylureas, α-glucosidase inhibitors, according to local labels (dipeptidyl peptidase-4 inhibitors and other incretin based therapies are not allowed)
  - Insulin therapy should be based on local practice, including basal, basal/bolus, premix, and mealtime bolus (SIT)

Blood pressure
- Target: 130/80 mm Hg

Antihypertensive therapy
- First line: ACE inhibitors or ARBs
- Based on individual patient needs: Ca2+–blockers, diuretics, others

Lipid targets and therapy
- LDL <100 mg/dL (<70 mg/dL in patients with previous cardiovascular events)
- Statins recommended for all patients
- Second line therapy at investigator discretion

Antiplatelet therapy
- Aspirin or clopidogrel (if aspirin intolerant) for patients with prior cardiovascular events (MI, cerebrovascular accident, or revascularization)

For patients with suboptimal glucose control after randomization, concomitant use and dosage of insulin, sulfonylureas, glimepiride, thiazolidinediones, and α-glucosidase inhibitors is permitted at the discretion of the investigator, but use of other GLP-1 agonists, dipeptidyl peptidase-4 inhibitors, and pramlintide is not.

Concomitant use of premixed insulin. Continued use of premixed insulin, including injection frequency and timing, during the trial is permitted at the investigator's discretion. A 20% reduction in insulin dosage when starting randomized therapy is recommended for patients with HbA1c ≤8%.

The LEADER global expert panel (GEP) and national study leaders in participating countries developed a protocol for the treatment of risk factors and concomitant use of medications. Guidelines were finalized during a series of workshops using consensus practice recommendations in 2010.[13-16] Table II contains a list of the finalized standard of care guidelines endorsed by the LEADER steering committee.

Planned follow-up. After randomization, patients were initially seen at 1, 3, and 6 months. Thereafter, patients are seen every 6 months for up to 5 years. During each study visit, patients are assessed for clinical events, study drug compliance, and concomitant medication

usage. Blood, urine specimens, and electrocardiograms were collected at randomization and then yearly for the duration of the study.

End points. The primary end point is the time from randomization to a composite outcome consisting of the first occurrence of cardiovascular death, nonfatal MI, or nonfatal stroke. Comprehensive descriptions for each component of the primary composite end point are listed in the online Appendix Supplementary Table I. Secondary end points include the first occurrence of an expanded composite cardiovascular outcome, including cardiovascular death, nonfatal MI, nonfatal stroke, revascularization, hospitalization for unstable angina, or hospitalization for chronic heart failure. Additional end points include time from randomization to the occurrence of noncardiovascular or all-cause death, each individual component of the expanded composite cardiovascular outcome, composite microvascular outcomes, and each individual component of composite microvascular outcomes.

Safety end points. Additional end points are being assessed to support the secondary efficacy and safety objectives (online Appendix Supplementary Table II). Hypoglycemia is defined according to American Diabetes Association criteria[17] as (1) severe: requiring the assistance of another person to administer resuscitative actions, carbohydrate, or glucagon; (2) documented symptomatic: typical symptoms of hypoglycemia accompanied by a measured plasma glucose concentration ≤70 mg/dL; (3) asymptomatic: measured plasma glucose concentration ≤70 mg/dL in the absence of symptoms; (4) probable symptomatic: unmeasured plasma glucose concentration in the presence of typical symptoms of hypoglycemia; and (5) relative hypoglycemia: typical symptoms of hypoglycemia and interpreted by the patient as a hypoglycemic episode with a measured plasma glucose concentration ≥70 mg/dL. An independent, event adjudication committee (EAC) blinded to treatment arm will adjudicate serious adverse events, including pancreatitis or severe persistent abdominal pain leading to suspicion of pancreatitis, neoplasm, and thyroid disease resulting in thyroidectomy (online Appendix Supplementary Table II).

Calcitonin monitoring. Blood samples were collected at screening to assess baseline levels of calcitonin in a central laboratory; patients with values ≥50 ng/L were excluded. Calcitonin levels are measured at prespecified time intervals in all subjects throughout the study. An independent calcitonin-monitoring committee (CMC) consisting of thyroid experts blinded to treatment provides ongoing surveillance to monitor longitudinal changes in calcitonin levels. Any value ≥20 ng/L is reported to the CMC for review and consideration of additional diagnostic procedures. For any level ≥20 ng/L, testing is repeated within 4 weeks. If the level is confirmed, a medical event of special interest is reported, and the CMC provides guidance on individualized follow-up and/or subsequent monitoring to site investigators. All medical events of special interest, including development of neoplasm or thyroid disease resulting in thyroidectomy, are reviewed and adjudicated by the EAC.

Study organization, event adjudication, and data monitoring. LEADER is overseen by a steering committee composed of experts in endocrinology, cardiology, gastroenterology, thyroid disease, nephrology, biostatistics, and employees of the study sponsor (Novo Nordisk). The steering committee independently oversees all aspects of the trial. The GEP consists of principal investigators from enrolling countries and designated employees of the sponsor and provides advice and active implementation assistance for operational issues.

Several end points will be adjudicated by the EAC based on several efficacy end points, including death, acute coronary syndrome (MI and hospitalization for unstable angina), cerebrovascular events (stroke and transient ischemic attack), coronary revascularization procedures, hospitalization for heart failure, nephropathy, and diabetic retinopathy.

The EAC consists of experts in cardiology, neurology, gastroenterology, endocrinology, oncology, pathology, nephrology, and ophthalmology and will meet throughout the duration of the trial. A chairperson oversees 4 separate subcommittees consisting of 16 primary adjudicators. The cardiovascular subcommittee includes 2 cardiologists and 2 neurologists. The microvascular subcommittee includes 2 nephrologists and 2 ophthalmologists. The pancreatitis subcommittee includes 3 gastroenterologists. The neoplasm subcommittee includes 3 oncologists and 2 endocrinologists. In case of thyroid disease resulting in a thyroidectomy, adjudicators include 1 endocrinologist and 1 oncologist who will review both the local pathology report and the report of an external pathologist who has reviewed the pathology specimen independently.

An independent, external data-monitoring committee (DMC) was established to perform ongoing safety surveillance and consists of permanent members who are recognized experts in cardiology, endocrinology, gastroenterology, and statistics. The DMC has access to complete unblinded data and meets at predefined intervals and on an ad hoc basis as required to evaluate all relevant safety information. After each meeting, the DMC will issue a recommendation on trial continuation, modification, or termination. The DMC can recommend to terminate the trial prematurely in case there is evidence for an excess number of deaths in the liraglutide group (significance level determined at $P < .01$), an excess number of major adverse cardiovascular events in the liraglutide group (significance level determined at $P <$

Figure 2

Enrollment of LEADER trial participants by country. Data presented are number of randomized participants/number of trial sites that randomized participants by country. In total, 9,340 participants were randomized across 410 active LEADER trial sites. There were 7 sites that screened but did not enroll or randomize any participants.

.01), or clear evidence of benefit for the primary end point in the liraglutide arm (significance level determined at $P < .001$).

Funding

The LEADER trial was funded by Novo Nordisk. No extramural funding was used to support the creation of this article. The authors are solely responsible for the design and conduct of this study, all study analyses, and the drafting and editing of the paper and its final contents.

Statistical considerations

Sample size calculation. The required sample size was estimated on the basis of time to first primary outcome using a log-rank test that included the full analysis set and an intention-to-treat principle. The primary event rate was estimated to be 1.8% in both the liraglutide and placebo groups, with uniform enrollment over 1.5 years, and a maximum follow-up period of 5 years. The noninferiority margin was set at 1.3 for the upper bound of the 2-sided 95% CI. It was also estimated that patients who permanently stop randomized treatment and are lost to follow-up would not exceed 10% in total. Finally, the power to reject the null hypothesis (ie, that the upper bound of the 2-sided CI would exceed 1.3) was set at 90%. Given these assumptions, 8,754 randomized subjects were required with an accrual of no <611 events with a minimum follow-up of 42 months after last subject randomized.

Analysis of the primary end point. Only outcomes confirmed by the blinded EAC will be analyzed. Subjects who complete or discontinue the trial without having an outcome will be censored for the relevant analyses on the last day of follow-up. The primary end point will be analyzed for the full analysis set and performed using Cox regression, including only treatment group as a covariate. The Cox regression model will be used to estimate the hazard ratio (liraglutide to placebo) and the 2-sided 95% CI. The objective of the LEADER trial is to assess the cardiovascular safety of liraglutide. Safety will be established if the two-sided 95% confidence limit is less than the prespecified upper bound of 1.3, as established by the United States Food and Drug Administration. If safety is established, then formal superiority testing will be performed. Noninferiority of liraglutide versus placebo will be assessed and established if the upper limit is <1.3. If noninferiority is established for the primary outcome, the data will be used to test for evidence of a lower outcome hazard with liraglutide versus placebo. Superiority with respect to the hazard ratio will be established if the upper range

Table III. Baseline characteristics of all randomized subjects and by presence of existing CVD

Clinical demographics	Previous CVD (*n* = 7,592)	No previous CVD (*n* = 1,748)	Total (*N* = 9,340)
Age, y	63.9 ± 7.6	65.8 ± 5.2	64.3 ± 7.2
Gender (male)	5,048 (66.5)	955 (54.6)	6,003 (64.3)
Ethnicity			
Hispanic or Latino	908 (12.0)	227 (13.0)	1,135 (12.2)
Not Hispanic or Latino	6,684 (88.0)	1,521 (87.0)	8,205 (87.8)
Race			
White	5,974 (78.7)	1,263 (72.3)	7,237 (77.5)
Asian	753 (9.9)	169 (9.7)	922 (9.9)
Black	535 (7.0)	240 (13.7)	775 (8.3)
Other	330 (4.3)	76 (4.3)	406 (4.3)
Weight, kg	92.3 ± 20.9	89.6 ± 21.4	91.8 ± 21.0
Body mass index, kg/m^2	32.5 ± 6.3	32.4 ± 6.3	32.5 ± 6.3
Hypertension	6,888 (90.7)	1,520 (87.0)	8,408 (90.0)
Hyperlipidemia	6,135 (80.8)	1,056 (60.4)	7,191 (77.0)
Smoking			
Current	927 (12.2)	203 (11.6)	1,130 (12.1)
Previous	3,670 (48.3)	667 (38.2)	4,337 (46.4)
Coronary artery disease	5,288 (69.7)	17 (1.0)	5,303 (56.8)
Congestive heart failure	1,562 (20.6)	37 (2.1)	1,599 (17.1)
Peripheral artery disease	1,394 (18.4)	250 (14.3)	1,644 (17.6)
Diabetes duration, y	12.8 ± 8.1	12.3 ± 7.5	12.7 ± 8.0
HbA1c, %	8.7 ± 1.5	8.8 ± 1.6	8.7 ± 1.5
Glucose-lowering therapy			
None/diet	405 (5.3)	99 (5.7)	504 (5.4)
Oral antihyperglycemics*			
1	1,539 (20.3)	378 (21.6)	1,917 (20.5)
2	2,131 (28.1)	555 (31.8)	2,686 (28.8)
≥3	257 (3.4)	71 (4.1)	328 (3.5)
Insulin†	3,260 (42.9)	645 (36.9)	3,905 (41.8)
Aspirin use	5,807 (76.5)	716 (41.0)	6,523 (69.8)
Laboratory Evaluation			
Total cholesterol, mg/dL	168.5 ± 45.4	178.8 ± 43.8	170.4 ± 45.3
LDL cholesterol, mg/dL	88.0 ± 35.5	96.5 ± 34.6	89.5 ± 35.5
HDL, mg/dL	44.9 ± 12.1	48.0 ± 12.7	45.5 ± 12.3
Triglycerides, mg/dL	183.5 ± 141.1	177.8 ± 135.0	182.5 ± 140.0
Creatinine, mg/dL	1.0 ± 0.5	0.8 ± 0.2	1.0 ± 0.4
eGFR, mL/min/1.73m^2			
<30	177 (2.3)	0	177 (1.9)
30-60	1,854 (24.4)	0	1,854 (19.9)
60-90	2,942 (38.8)	918 (52.5)	3,860 (41.3)
>90	2,619 (34.5)	828 (47.4)	3,447 (36.9)
Lipase U/L	48.2 ±45.6	44.9 ± 34.6	47.5 ± 43.7
Amylase U/L	66.5 ± 36.8	64.7 ± 33.7	66.2 ± 36.3

Data presented as number (percentage of group) or mean ± SD.
*Not used in combination with insulin.
† Either used alone or in combination with concomitant oral antihyperglycemics.

of the two-sided 95% CI is <1. This approach is a closed testing procedure, and therefore, no adjustment of the significance level is required.[18,19]

Exploratory subgroup analyses. The effect of sex, age (<60 or ≥60 years), body mass index (≤30or >30 kg/m^2), HbA1c (≤8.3 or >8.3%), duration of diabetes (≤11 or >11 years), region (Europe, North America, Asia, or other), race (white, black, Asian, or other), cardiovascular risk, chronic heart failure, severe chronic renal failure, severe-to-moderate chronic renal failure, and use of concomitant glucose medication and/or insulin on the primary composite end point will be explored separately as a main effect and interaction with treatment by adding each to the original model.

There will be 2 populations analyzed. The full analysis set includes all randomized subjects with evaluation by intention-to-treat, and subjects will be evaluated as randomized. A sensitivity analysis will be performed using the per-protocol analysis set that includes only data from follow-up of subjects exposed to treatment plus 30 days. Subjects exposed to treatment in the per-protocol analysis will include those with a maximum accumulated drug holiday of ≤120 days during the study. Subjects accumulating >120 days will be

considered as having discontinued study drug on the 121st day.

Enrolled population

Enrollment for LEADER commenced in September 2010 and concluded in April 2012. Patients were enrolled at 410 sites in 32 countries (Figure 2). Baseline demographics of the enrolled population are shown in Table III. Of the 9,340 patients, 7,592 (81.3%) had prior CVD and 1,748 (18.7%) did not. There were 1,854 patients with moderate and 177 with severe reductions in screening eGFR.

Discussion

There remains a compelling need to develop novel, effective, and safe glucose-lowering therapies for patients with T2DM. Liraglutide is associated with a reduction in HbA1c ranging from 1.0 to 1.5%.[20] Current dosing of liraglutide was derived from clinical data aiming to improve gastrointestinal tolerability while maintaining efficacy. From this perspective, a starting dose of 0.6 mg daily is suggested, increasing to 1.2 or 1.8 mg based on clinical response.

Prior studies have investigated the perceived beneficial effects of GLP-1 receptor agonists on cardiovascular risk. The mechanism of action behind these effects remains to be clarified. However, there appear to be a number of direct and indirect effects of treatment possibly explaining this reduction in risk, such as significant weight and systolic blood pressure reduction and, potentially, direct effects on cardiac myocytes and endothelium.[21] A decrease in systolic blood pressure of 2.1 mm Hg with liraglutide 1.2 mg and 3.6 mm Hg with 1.8 mg, together with a sustained mean weight loss of approximately 2 kg, has been reported.[22] In addition, data from 5 long-term phase 3 trials suggested no adverse impact of liraglutide treatment on lipid profiles with respect to cardiovascular risk and favorable changes in triglycerides and free fatty acids. Others have reported decreases in levels of cardiovascular risk markers such as plasminogen-activator inhibitor-1 and B-natriuretic peptide after treatment.[23-25] However, liraglutide is associated with an approximate 1–2 beat/min increase in heart rate.

Conclusions

LEADER is a phase 3B randomized, double-blind clinical trial to evaluate the cardiovascular safety of liraglutide in patients with T2DM at heightened risk for cardiovascular complications. It is expected that LEADER will provide conclusive data regarding the cardiovascular safety of liraglutide relative to standard of care for a global population of patients with T2DM.

Disclosures

Conflicts of interest: Dr Bergenstal has served on a scientific advisory board, consulted or performed clinical research with Abbott Diabetes Care, Amylin, Bayer, Becton Dickinson, Boehringer Ingelheim, Bristol-Myers Squibb/AstraZeneca, Intuity, Calibra, DexCom, Eli Lilly, Halozyme, Helmsley Trust, Hygieia, Johnson & Johnson, Medtronic, Merck, NIH, Novo Nordisk, ResMed, Roche, Sanofi, and Takeda. His employer, nonprofit Park Nicollet Institute, contracts for his services, and no personal income goes to Dr Bergenstal. He has inherited stock in Merck.

Dr Buse is an investigator and/or consultant without any direct financial benefit under contracts between his employer and the following companies: Abbott, Amylin, Andromeda, AstraZeneca, Bayhill Therapeutics, BD Research Laboratories, Boehringer-Ingelheim, Bristol-Myers Squibb, Catabasis, Cebix, Diartis, Elcylex, Eli Lilly, Exsulin, Genentech, GI Dynamics, GlaxoSmithKline, Halozyme, Hoffman-LaRoche, Johnson & Johnson, LipoScience, Medtronic, Merck, Metabolic Solutions Development Company, Metabolon, Novan, Novartis, Novo Nordisk, Orexigen, Osiris, Pfizer, Rhythm, Sanofi, Spherix, Takeda, Tolerex, TransPharma, Veritas, and Verva.

Dr Brown Frandsen is a full-time employee of and holds stock in Novo Nordisk A/S.

Dr Daniels is a consultant for Genzyme (Sanofi), Exilixis, and Novo Nordisk.

Dr Mann is an investigator and/or consultant receiving honoraria from Abbott, Bayer, Boehringer-Ingelheim, Novo-Nordisk, Roche, and Vifor.

Dr Marso reports no personal conflicts of interest during the previous 12 months. All compensation for his research activities, including research grants and consulting fees from The Medicines Company, Novo Nordisk, Abbott Vascular, Amylin Pharmaceuticals, Volcano Corporation, St. Jude Medical, and Terumo Medical, are paid directly to the Saint Luke's Hospital Foundation of Kansas City.

Dr Moses is a full-time employee of and holds stock in Novo Nordisk A/S.

Dr Nissen reports that the Cleveland Clinic Center for Clinical Research receives funding to perform clinical trials from Amgen, Pfizer, Novartis, Takeda, Resverlogix, Ethicon, Orexigen, Vivus, and Eli Lilly. Dr Nissen is involved in these clinical trials but receives no personal remuneration for his participation. Dr Nissen consults for many pharmaceutical companies, including Novo Nordisk, but requires them to donate all honoraria or consulting fees directly to charity so that he receives neither income nor a tax deduction.

Dr Nauck has received research grants payable to his institution, the Diabeteszentrum Bad Lauterberg, from Berlin-Chemie/Menarini, Eli Lilly, Merck, Sharp & Dohme, Novartis Pharma, AstraZeneca, Boehringer Ingelheim,

GlaxoSmithKline, MetaCure, Roche Pharma, Novo Nordisk Pharma, Tolerx Inc; consulting fees and/or honoraria for membership in advisory boards and/or honoraria for speaking from Amylin Pharmaceuticals, AstraZeneca, Berlin-Chemie/Menarini, Boehringer Ingelheim, Bristol-Myers Squibb, Diartis Pharmaceuticals, Eli Lilly, Hoffmann-LaRoche, GlaxoSmithKline, Intarcia Therapeutics, MannKind Corp, Merck, Sharp & Dohme, Novartis Pharma, Novo Nordisk, Sanofi, Takeda, and Wyeth Research, including reimbursement for travel expenses in connection with the previously mentioned activities. He owns no stock and is employed by Diabeteszentrum Bad Lauterberg, Germany.

Dr Pocock reports receiving honoraria for serving on independent data monitoring committees for the EXSCEL, TECOS, and ACE trials.

Dr Poulter has received financial support from several pharmaceutical companies that manufacture either blood pressure–lowering or lipid-lowering agents, or both, for consultancy fees, research projects and staff, and for arranging and speaking at educational meetings. He holds neither stock nor shares of stock in any such companies.

Dr Steen Ravn is a full-time employee of and holds stock in Novo Nordisk A/S.

Dr Steinberg has served as a legal consultant for Eli Lilly, Amylin, and Novo Nordisk.

Dr Zinman has received honoraria from Novo Nordisk for scientific advisory board meetings and presentations. His institution has received research support from Novo Nordisk.

Funding for the LEADER Trial is provided by Novo Nordisk.

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Appendix

Supplementary Table I. Definitions Used for Clinical Events

Event	Definition
Cardiovascular death (occurring from any of the following):	**Sudden, unexpected cardiac cause:** • Cardiac arrest (symptomatic myocardial ischemia, new ST elevation or left bundle branch block, and/or angiographic or autopsy evidence of new thrombus) **CV cause** • Sudden cardiac death by acute MI, heart failure, stroke, other cardiovascular causes, or no documented nonvascular cause **Sudden cardiac cause**: Unexpected occurrence in a previously stable patient and including: • Absence of new or worsening symptoms • Within 60 min of new/worsening symptom onset • Attributed to identified arrhythmia on ECG or witnessed by emergency medical technicians • Unsuccessful resuscitation from cardiac arrest or resuscitation but death within 24 h without noncardiac etiology • Other cause with information on clinical status within the week preceding death **Acute MI**: • Occurring up to 30 days after documented acute MI and no conclusive evidence of other cause • Occurring before biomarker confirmation of myocardial necrosis with adjudication based on clinical presentation and ECG evidence **Other cardiovascular cause** • MI occurring directly from cardiovascular procedures **Heart failure or cardiogenic shock** Occurring in the context of clinically worsening symptoms and/or signs of heart failure without evidence of another cause of death. New or worsening signs or symptoms without evidence of another cause including: • Requiring initiation or increase in treatment for heart failure or occurring in a patient on maximal therapy • Signs/symptoms requiring continuous intravenous therapy or oxygen administration • Confinement to bed for heart failure symptoms • Pulmonary edema sufficient to cause tachypnea and distress not occurring in an acute MI or as the consequence of arrhythmia without worsening heart failure • Cardiogenic shock not occurring in an acute MI or from an arrhythmia in the absence of worsening heart failure. Cardiogenic shock: systolic blood pressure <90 mm Hg >1 h, unresponsive to fluids or heart rate correction, secondary to cardiac dysfunction and associated with >1 of the following: • Cool, clammy skin or • Oliguria (urine output <30 mL/h) • Altered sensorium • Cardiac index <2.2 L min^{-1} m^{-2} • SBP ≥90 mm Hg from positive inotropic or vasopressor agents alone and/or with mechanical support in <1 h, and after randomization. • Occurring before and continuing after randomization not included • Sudden death during admission for worsening heart failure. **Cerebrovascular event**: • Intracranial hemorrhagic or nonhemorrhagic stroke occurring up to 30 d and based on clinical signs and symptoms, neuroimaging or autopsy, and no conclusive evidence of other cause of death **Other cardiovascular cause** **Presumed cardiovascular cause** Not attributed to cardiovascular or noncardiovascular are presumed cardiovascular deaths
Myocardial infarction	Any of the following based European Society of Cardiology/American College of Cardiology Foundation)/American Heart Association/World Heart Federation criteria[25]: • **Spontaneous MI:** Rise and/or fall in cardiac biomarkers with >1 value >ULN and evidence of: o Ischemia o New ischemia on ECG ST-T changes or left bundle branch block*) o Pathological Q waves on ECG† o New loss of viable myocardium or regional wall motion abnormality on imaging • **ST-elevation MI**: At the J point in 2 contiguous leads (≥0.2 mV in men or ≥0.15 mV in women in leads V2-V3 and/or ≥0.1 mV in other leads)

(*continued on next page*)

Supplementary Table I. (*continued*)

	• **Non-ST-elevation MI:** At the J point in 2 contiguous leads (≥0.2 mV in men or ≥0.15 mV in women in leads V2-V3 and/or ≥0.1 mV in other leads absent on ECG) • **PCI-Related MI**: Elevations of cardiac biomarkers >99th percentile upper reference limit in patients with normal baseline troponin values **OR**
	○ In patients with elevated cardiac biomarkers before PCI, a ≥20% increase in a second biomarker sample within 24 h of PCI and documented decreasing values before suspected recurrent MI • **Coronary artery bypass grafting-related mi:** Normal baseline troponin values with elevated cardiac biomarkers >99th percentile URL ○ In patients with elevated cardiac biomarkers before CABG, a ≥20% increase in a second biomarker sample within 72 h of CABG and documented decreasing values before suspected recurrent MI and either new pathological Q waves in at least 2 contiguous leads on ECG or new left bundle branch block, angiographically documented new graft or native coronary artery occlusion, or loss of viable myocardium on imaging • **Silent MI:** ○ No evidence of acute MI **AND** New pathological Q waves, evidence a regional loss of viable myocardium on imaging, evidence of healed or healing MI on autopsy
Cerebrovascular events (stroke and TIA)	**Stroke:** Acute neurologic dysfunction documented by CT, MRI, or autopsy and attributed to a vascular cause and determined to *not* be due to readily identifiable cause, **Transient ischemic attack** <24 h **Micro-hemorrhage:** Rounded <5 to 10 mm foci of susceptibility artifact on MRI . (occurrence not included in the primary event)
Classification of cerebrovascular events (stroke and TIA)	**Transient ischemic attack:** Neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia, without acute infarction. **Ischemic stroke**: Acute episode of focal cerebral, spinal, or retinal dysfunction caused by an infarction of central nervous system tissue **Hemorrhagic stroke**: Acute episode of focal or global cerebral, spinal, or retinal dysfunction caused by a nontraumatic intraparenchymal, intraventricular, or subarachnoid hemorrhage with documentation of cerebral hemorrhage on imaging or autopsy **Undetermined stroke**: Insufficient information to categorize

* ECG manifestations of acute myocardial ischemia (in absence of left ventricular hypertrophy [LVH] and left bundle branch block (LBBB): (1) ST elevation New ST elevation at the J point in 2 contiguous leads with the cutoff points: ≥0.2 mV in men or ≥0.15 mV in women in leads V2-V3 and/or ≥0.1 mV in other leads. (2) ST depression and T-wave changes New horizontal or down-sloping ST depression ≥0.05 mV in 2 contiguous leads; and/or T inversion ≥0.1 mV in 2 contiguous leads with prominent R wave or R/S ratio >1.
† Pathological Q waves: (1) Any Q wave in leads V2-V3 ≥0.02 s or QS complex in leads V2 and V3 Q wave ≥0.03 s and ≥0.1 mV deep or QS complex in leads I, II, aVL, aVF, or V4-V6 in any 2 leads of a contiguous lead grouping (I, aVL, V6; V4-V6; II, III, and aVF).

Supplementary Table II. Other efficacy and safety end points

Time from randomization to first occurrence of a composite microvascular outcome:
- Retinal photocoagulation
- Vitreous hemorrhage
- Diabetes-related blindness
- New or worsening nephropathy (defined as new onset of macroalbuminuria, or doubling of serum creatinine level and eGFR ≤45 mL/min per 1.73m^2, or the need for continuous renal-replacement therapy (in the absence of an acute reversible cause)
- Death due to renal disease

Diabetic foot ulcers

Change from baseline to the last assessment during the treatment period in:
- Weight and waist circumference
- HbA1c
- Blood lipids: total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides
- Blood pressure and pulse rate
- eGFR rate (MDRD and chronic kidney disease-EPI formulas)
- Laboratory parameters:
  - Lipase
  - Amylase
  - Calcitonin
  - Anti liraglutide antibodies
  - ALT
  - Bilirubin (total)
  - Calcium (total)
  - Sodium
  - Potassium
  - Urinary albumin to creatinine ratio

Change from baseline to assessment at 3 y during the treatment period in:
- Weight and waist circumference
- HbA1c
- Blood lipids: total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides
- Blood pressure and pulse rate
- eGFR rate (MDRD and chronic kidney disease-EPI formulas)
- Incidence of hypoglycemic episodes

Incidence of serious adverse events and medical events of special interest:
- Neoplasm
- Pancreatitis
- Acute, severe and persistent abdominal pain leading to a suspicion of pancreatitis
- Acute gallstone disease (biliary colic or acute cholecystitis)
- First confirmed episode of calcitonin concentration increase ≥20 ng/L
- Thyroid disease
- Severe hypoglycemic event
- Immunogenicity event (antibody formation, allergic reactions, immune complex disease and injection site disorders)
- Adverse events leading to treatment discontinuation

Patient reported outcome assessed by EQ-5D questionnaire (in a subset of subjects only)

Supplementary Table III. Principal Investigators by Country

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EXHIBIT 30

Beneficial Endocrine but Adverse Exocrine Effects of Sitagliptin in the Human Islet Amyloid Polypeptide Transgenic Rat Model of Type 2 Diabetes

Interactions With Metformin

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OBJECTIVE—We sought to establish the extent and mechanisms by which sitagliptin and metformin singly and in combination modify islet disease progression in human islet amyloid polypeptide transgenic (HIP) rats, a model for type 2 diabetes.

RESEARCH DESIGN AND METHODS—HIP rats were treated with sitagliptin, metformin, sitagliptin plus metformin, or no drug as controls for 12 weeks. Fasting blood glucose, insulin sensitivity, and β-cell mass, function, and turnover were measured in each group.

RESULTS—Sitagliptin plus metformin had synergistic effects to preserve β-cell mass in HIP rats. Metformin more than sitagliptin inhibited β-cell apoptosis. Metformin enhanced hepatic insulin sensitivity; sitagliptin enhanced extrahepatic insulin sensitivity with a synergistic effect in combination. β-Cell function was partially preserved by sitagliptin plus metformin. However, sitagliptin treatment was associated with increased pancreatic ductal turnover, ductal metaplasia, and, in one rat, pancreatitis.

CONCLUSIONS—The combination of metformin and sitagliptin had synergistic actions to preserve β-cell mass and function and enhance insulin sensitivity in the HIP rat model of type 2 diabetes. However, adverse actions of sitagliptin treatment on exocrine pancreas raise concerns that require further evaluation.
***Diabetes* 58:1604–1615, 2009**

The prevalence of type 2 diabetes and the associated morbidity and mortality are increasing (1). There is therefore interest in strategies to slow or prevent the development of type 2 diabetes. Although insulin resistance secondary to lifestyle changes likely contributes to the increased prevalence, most insulin-resistant individuals increase insulin secretion and remain nondiabetic (2). In contrast, in those genetically vulnerable to develop type 2 diabetes, β-cell function fails to appropriately adapt to insulin resistance, leading to hyperglycemia (3,4).

Prospective studies in humans have reported a progressive decline in β-cell function preceding development of type 2 diabetes (5,6). Autopsy studies reveal that the islet in type 2 diabetes is characterized by a ~60% deficit in β-cells and islet amyloid derived from islet amyloid polypeptide (IAPP), a 37–amino acid peptide cosecreted with insulin by β-cells (7). The cause of the defect in β-cell mass in type 2 diabetes remains unresolved but is likely attributable, at least in part, to endoplasmic reticulum stress–induced β-cell apoptosis, noted both at autopsy and in isolated islets from people with type 2 diabetes (8,9). Based on these observations, it is apparent that to favorably modify disease progression in type 2 diabetes, preservation of β-cell mass and function in the setting of insulin resistance is required.

Our primary objective in the current study was to test the hypothesis that the combination of two potentially synergistic therapies, the dipeptidyl peptidase-4 (DPP-4) inhibitor sitagliptin and hepatic insulin sensitizer metformin, modify progression of islet dysfunction and loss of β-cell mass in type 2 diabetes. Because it is not possible to evaluate β-cell mass or turnover in vivo in humans, we undertook these studies in the human IAPP transgenic (HIP) rat because it approximates the islet and metabolic phenotype of type 2 diabetes in humans (10–12).

Metformin has previously been shown to delay onset of type 2 diabetes (13). Glucagon-like peptide 1 (GLP-1) has reversed loss of β-cell mass in some murine models of diabetes by both increasing new β-cell formation and decreasing β-cell apoptosis (14–16). The DPP-4 inhibitor sitagliptin increases GLP-1 concentrations (17) and modestly lowers glucose levels when used alone in type 2 diabetes (18,19) with an additive effect in combination with metformin (20,21).

Therefore, we sought to address the following questions. First, do metformin or sitagliptin individually or in combination favorably modify disease progression (reducing β-cell loss and dysfunction) at the level of the islet in the HIP rat model of type 2 diabetes? Second, is any protection of β-cell mass accomplished by decreased β-cell apoptosis and/or increased β-cell formation? Third, what are the respective actions of these drugs on insulin sensitivity and secretion singly, and in combination, in this model of type 2 diabetes? Unexpectedly, we encountered marked ductal metaplasia in 25% of high-fat diet–fed HIP rats treated with sitagliptin and severe hemorrhagic pancreatitis in one sitagliptin-treated animal. Because those

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Received 12 January 2009 and accepted 8 April 2009.
Published ahead of print at http://diabetes.diabetesjournals.org on 29 April 2009. DOI: 10.2337/db09-0058.

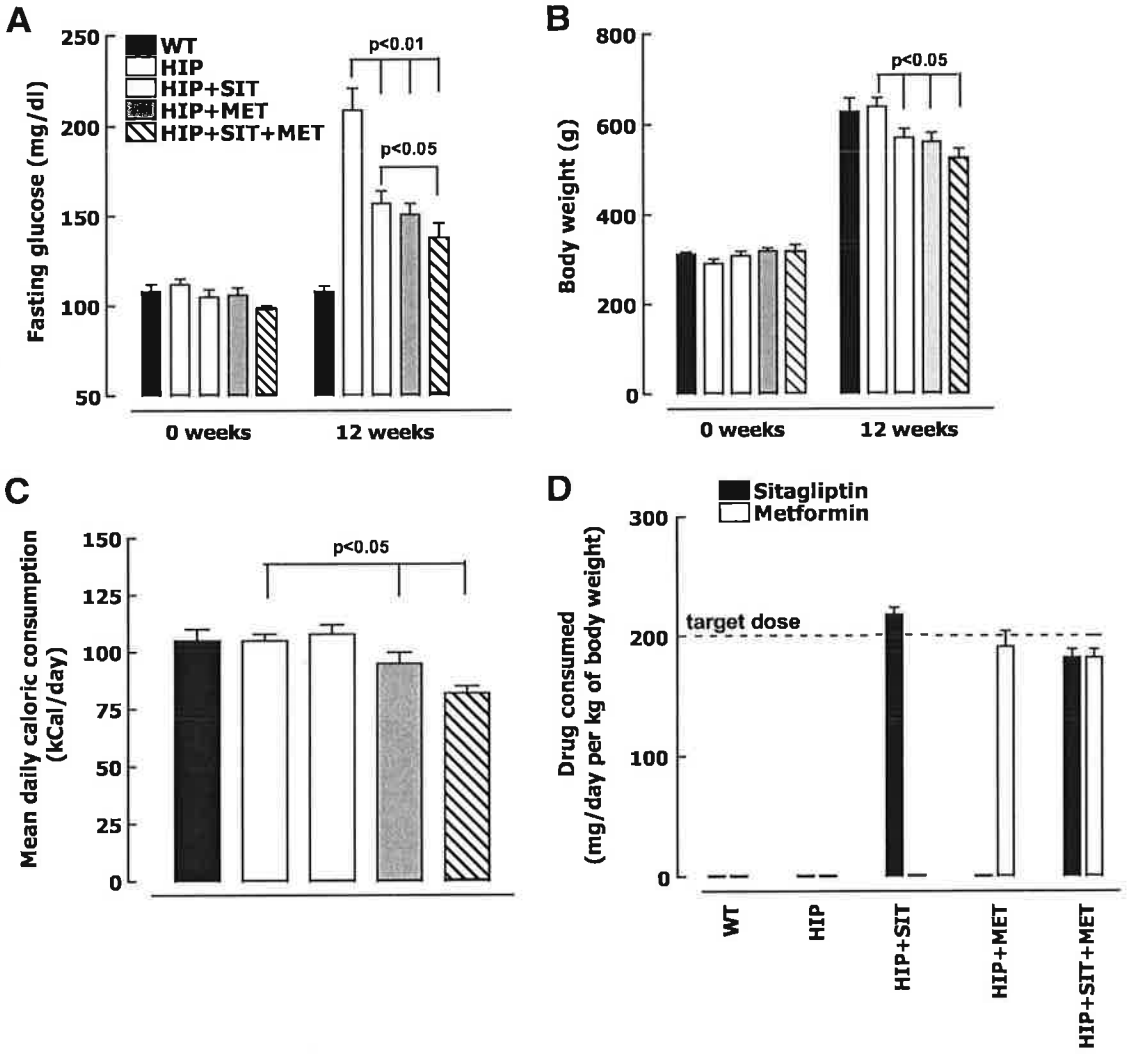

FIG. 1. Fasting plasma glucose (*A*), body weight (*B*), mean daily food intake (*C*), and mean daily drug consumption (*D*) after 12-week treatment with 60% high-fat chow diet in wild-type (WT) rats ($n = 7$), HIP rats ($n = 8$), HIP rats treated with sitagliptin (HIP+SIT; $n = 8$), HIP rats treated with metformin (HIP+MET; $n = 9$), and HIP rats treated with combination therapy (HIP+SIT+MET; $n = 8$). Data are means ± SE.

findings have potentially important clinical implications, we evaluated the exocrine effects of sitagliptin. These latter studies provided some insights into the reported association of GLP-1 mimetic therapy by exenatide (22) or liraglutide (23) and pancreatitis, and they provide some cautions about the potential long-term effects of GLP-1 mimetic therapy, including DPP-4 inhibition in diabetes.

RESEARCH DESIGN AND METHODS

A total of 40 Sprague-Dawley rats (wild type; $n = 7$) and rats expressing human IAPP (HIP rats; $n = 33$) were used in the current study. Generation of HIP rats has been described in detail previously (11). Rats were bred and housed individually throughout the study at the University of California Los Angeles animal housing facility and subjected to standard 12-h light/dark cycle. The University of California Los Angeles institutional animal care and use committee approved all surgical and experimental procedures. To establish the actions of sitagliptin and metformin singly and in combination on islet protection, 2-month-old wild-type and HIP rats were fed high-fat diet ad libitum for 12 weeks (60% fat, 20% protein, and 20% carbohydrates; no. D12492; Research Diets, New Brunswick, NJ) and randomly assigned into five independent treatment groups: wild-type rats (no drug treatment, $n = 7$), HIP rats (no drug treatment, $n = 8$), HIP rats given sitagliptin (200 mg · kg body wt^{-1} · day^{-1} sitagliptin, $n = 8$), HIP rats given metformin (200 mg · kg body wt^{-1} · day^{-1} metformin, $n = 9$), and HIP rats given sitagliptin plus metformin (200 mg · kg body wt^{-1} · day^{-1} sitagliptin and metformin, $n = 8$). Sitagliptin was provided by Merck Research (Rahway, NJ), and metformin was purchased from Toronto Research Chemicals (Toronto, Canada). Compounds were administered after premixing with high-fat diet, which was performed by Research Diets. After 12 weeks of diet/drug treatment, animals were anesthetized with isoflourane (2.5%) by inhalation until effect (Isoflourane Vapor 19.1; Summit Anesthesia, Portland, OR). Indwelling catheters were then inserted into the right internal jugular vein and left carotid artery for subsequent in vivo metabolic studies, as previously described (24). All catheters were filled with 100 units/ml heparin/saline solution, exteriorized to the back of the neck, and encased in the infusion harness (Instech, Plymouth Meeting, PA).

Hyperglycemic clamp and arginine bolus injection. To assess glucose- and arginine-stimulated insulin secretion, wild-type rats ($n = 6$), HIP rats ($n = 8$), HIP rats given sitagliptin ($n = 8$), HIP rats given metformin ($n = 6$), and HIP rats given sitagliptin plus metformin ($n = 6$) underwent a hyperglycemic clamp followed by an arginine bolus injection, as previously described (10). In brief, after a 30-min equilibration period (−30 to 0 min), plasma samples were taken for measurements of baseline fasting glucose and insulin. Thereafter, animals received an intravenous glucose bolus (375 mg/kg) followed by a variable 50% (wt/vol) glucose infusion to clamp arterial glucose at ~250 mg/dl (0–70 min). At $t = 60$ min, rats received a bolus injection of L-arginine solution (1 mmol/kg; Sigma, St. Louis, MO). Arterial blood samples (50 μl) were taken at baseline (−30 and 0 min), at 1 and 5 min, and every 15 min thereafter during

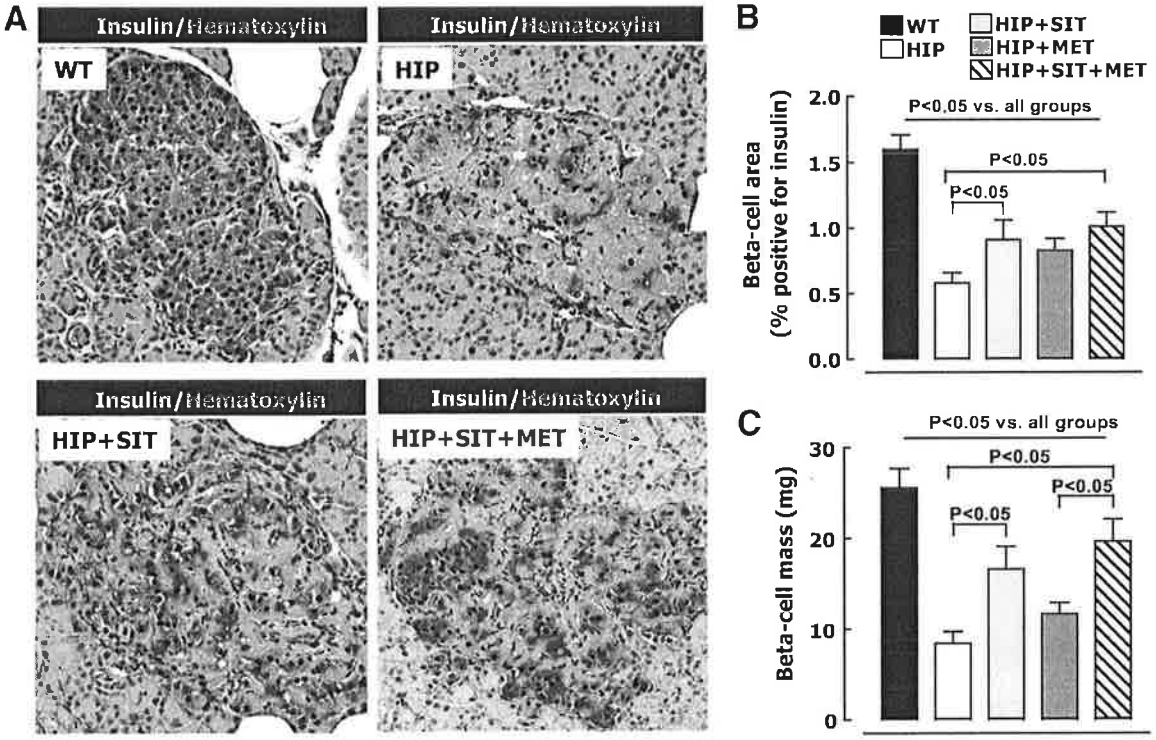

FIG. 2. *A*: **Typical islets from wild-type (WT) rats, HIP rats, HIP rats treated with sitagliptin (HIP+SIT), and HIP rats treated with sitagliptin and metformin (HIP+SIT+MET) stained for insulin (pink) and hematoxylin (blue). β-Cell area (*B*) and mean β-cell mass (*C*) after 12-week treatment with 60% high-fat diet in wild-type rats (n = 7), HIP rats (n = 8), HIP rats treated with sitagliptin (HIP+SIT; n = 8), HIP rats treated with metformin (HIP+MET; n = 9), and HIP rats treated with combination therapy (HIP+SIT+MET; n = 8). Data are the means ± SE. (A high-quality digital representation of this figure is available in the online issue.)**

the clamp for immediate determination of plasma glucose and subsequent analysis for insulin.

Hyperinsulinemic-euglycemic clamp and ^{3}H-glucose infusion. To assess insulin sensitivity and glucose turnover, wild-type rats (n = 5), HIP rats (n = 6), HIP rats given sitagliptin (n = 7), HIP rats given metformin (n = 6), and HIP rats given sitagliptin plus metformin (n = 6) underwent a hyperinsulinemic-euglycemic clamp with concomitant infusion of [3-^{3}H]glucose to assess glucose turnover, as previously described (10). Briefly, rats received primed (3 μCi) continuous (0.05 μCi/min) infusion of [3-^{3}H]glucose (Perkin Elmer, Boston, MA) for a 90-min basal period that increased to 0.2 μCi/min for 120 min throughout the hyperinsulinemic-euglycemic clamp, which was achieved by constant infusion of regular human insulin (Novolin; Novo Nordisk, Princeton, NJ) at 4 mU · kg^{-1} · min^{-1}, variable glucose (50% wt/vol) infusion, and somatostatin infusion (10 μg · kg^{-1} · min^{-1}; Bachem, CA) to inhibit endogenous insulin secretion. Plasma glucose levels were determined every 10 min, and additional blood samples (~100 μl) were collected at baseline (−30 min) and at the end of the clamp for determination of plasma insulin. Blood samples (~150 μl) for determination of tracer-specific activity were drawn at fasting (from −40 to 0 min) and during insulin infusion.

Endocrine pancreas histology. Rats were killed by 120 mg/kg i.v. sodium pentobarbital. The pancreas was then rapidly removed from killed rats and fixed in 4% paraformaldehyde overnight at 4°C. Paraffin-embedded pancreatic sections were stained first for hematoxylin/eosin and insulin (guinea pig anti-insulin, 1:100; Zymed, Carlsbad, CA). The β-cell mass was measured by first quantifying the pancreatic fractional area positive for insulin and multiplying this by the pancreatic weight. In addition, sections were costained by immunofluorescence for insulin (guinea pig anti-insulin, 1:100; Zymed, Carlsbad, CA) and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) method (Roche Diagnostics, Mannheim, Germany) for quantification of β-cell apoptosis, and they were costained for insulin (guinea pig anti-insulin, 1:100; Zymed) and Ki-67 (mouse anti–Ki-67, 1:50; Dako, Carpinteria, CA) for determination of β-cell replication. All β-cells per pancreatic section (~2,500 cells per section) were examined in detail and counted at ×200 magnification (×20 objective, ×10 ocular) for the total number of TUNEL- and Ki-67–positive β-cells. The frequency of TUNEL and Ki-67 was presented as the percentage of total β-cells per section. Fluorescent slides were analyzed and imaged using a Leica microscope (Leica Microsystems, Wetzlar, Germany) and images acquired using OpenLab microscope software (Improvision) and analyzed using ImagePro Plus software.

Exocrine pancreas histology. Pancreas sections were deparaffinized in xylene and rehydrated in ethanol gradient, and pancreatic sections were stained in Harris hematoxylin solution (HHS16; Sigma) and eosin Y solution (HT110132; Sigma). For immunofluorescence, antigen retrieval was performed via microwave heating in citrate buffer (H-3300; Vector, Burlingame, CA) except for TUNEL staining, which used proteinase-K digestion (V302B; Promega, Madison, WI) at 37°C for 15 min. Slides were blocked in Tris-buffered saline (3% BSA, 0.2% TX-100, and 2% donkey serum) for 1 h. The following primary antibodies were used for 12-h incubation: ductal cell marker cytokeratin (mouse anti-pancytokeratin, 1:50; Sigma), marker of cell fibrosis fibrinectin (rabbit anti-fibrinectin, 1:500; Sigma), replication marker Ki-67 (mouse anti–Ki-67, 1:50; Dako), apoptosis marker (TUNEL method; Roche Diagnostics), marker of T-cell infiltration (rabbit anti-CD3; Abcam, Cambridge, MA), marker of macrophage infiltration (rabbit anti–CD-11C; Abcam), GLP-1 receptor (rabbit anti–GLP-1 receptor, 1:100; Novus Biologicals, Littleton, CO), pancreatic and duodenal homeobox 1 (PDX-1) (rabbit anti–PDX-1, 1:1,000; Millipore, St. Louis, MO), and insulin (guinea pig anti-insulin, 1:100; Zymed). Secondary antibodies labeled with Cy3 and fluorescein isothiocyanate were obtained from Jackson Laboratories (West Grove, PA) and used at dilutions of 1:200 for 1-h incubation. To determine ductal cell replication and apoptosis, in each pancreatic section we quantified the total number of Ki-67–, TUNEL-, and cytokeratin-positive cells (~1,000 cytokeratin-positive cells per section were counted). The frequency of ductal cell replication and apoptosis in each animal was presented as a total number of TUNEL- or Ki-67–positive cells per total number of cytokeratin-positive cells.

Analytical procedures. Plasma glucose concentrations were measured by the glucose oxidase method (Glucose Analyzer 2; Beckman, Fullerton, CA). Plasma insulin was measured using competitive colorimetric enzyme-linked immunosorbent assay (Alpco Diagnostics, Salem, NH). Plasma glucose specific activity, hepatic glucose production, and glucose disposal was calculated as previously described in detail (10). Disposition index was calculated as the product of first-phase insulin secretion during the hyperglycemic clamp

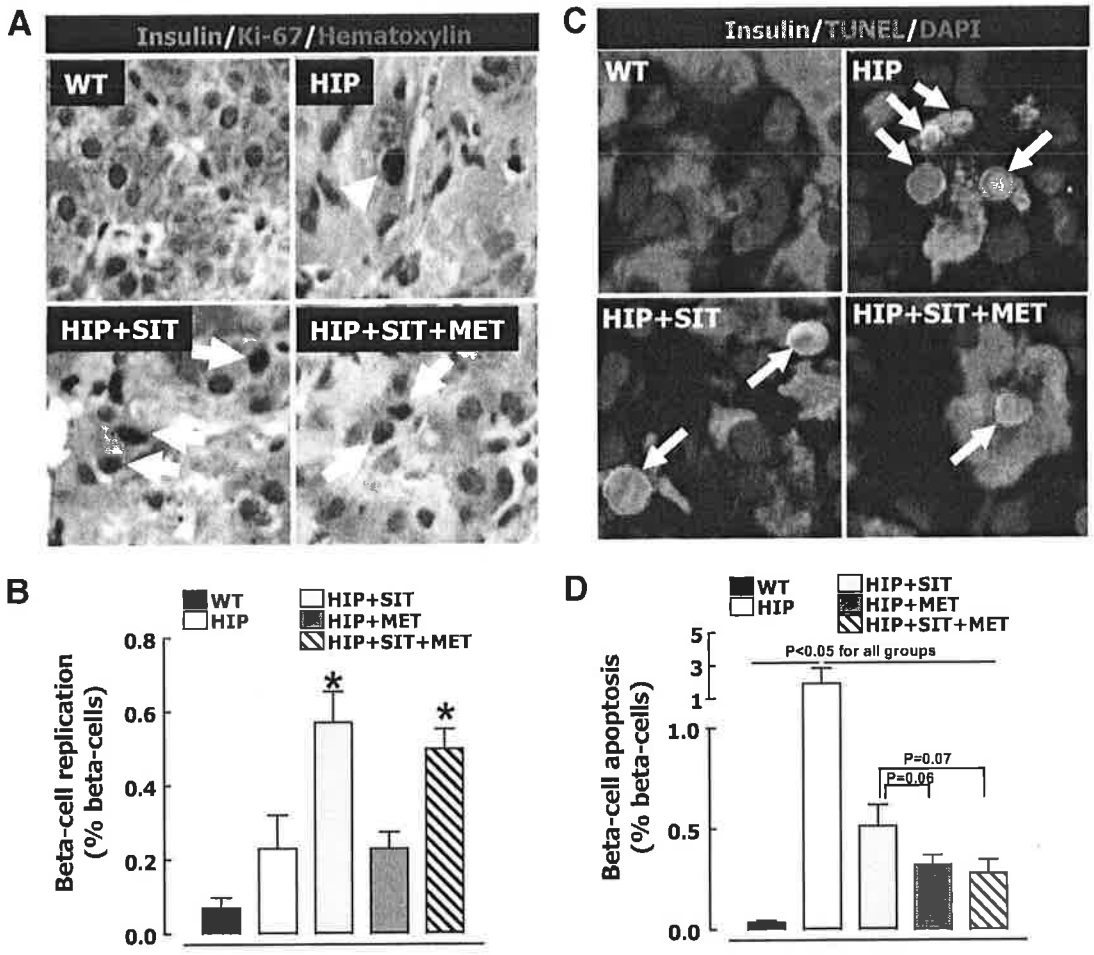

FIG. 3. ***A*: Examples of islets stained for insulin (pink) and replication marker Ki-67 (brown) and nuclear stain hematoxylin (blue) imaged at 20×. *B*: Frequency of β-cell replication in wild-type (WT) rats (n = 7), HIP rats (n = 8), HIP rats treated with sitagliptin (HIP+SIT; n = 8), HIP rats treated with metformin (HIP+MET; n = 9), and HIP rats treated with combination therapy (HIP+SIT+MET; n = 8). *C*: Examples of islets stained for insulin (green) and apoptosis marker (TUNEL; red) and nuclear stain (DAPI; blue) imaged at ×20. *D*: Frequency of β-cell apoptosis in wild-type rats (n = 7), HIP rats (n = 8), HIP rats treated with sitagliptin (n = 8), HIP rats treated with metformin (n = 9), and HIP rats treated with combination therapy (n = 8). Data are means ± SE. *P < 0.05 vs. wild type, HIP, and HIP plus metformin. Arrows indicate examples of insulin-positive Ki-67 and TUNEL-positive cells. (A high-quality digital representation of this figure is available in the online issue.)**

(expressed as pmol/l · min) and insulin sensitivity determined by mean glucose infusion rates (expressed in mg · kg^{-1} · min^{-1}) required to maintain euglycemia during the hyperinsulinemic-euglycemic clamp.

Statistical analysis. Statistical analysis was performed using ANOVA with Fisher's post hoc test where appropriate. Regression analysis was performed using Statistica (version 6; Statsoft, Tulsa, OK). Data in graphs and tables are the means ± SE. Findings were assumed to be statistically significant at P < 0.05.

RESULTS

Blood glucose concentrations, body weight, and food intake. Prior to initiation of high-fat diet, blood glucose was comparable (105 ± 4 mg/dl) in wild-type and HIP rats (Fig. 1*A*). After 12 weeks of high-fat diet, plasma glucose increased to 209 ± 12 mg/dl in HIP rats but was unchanged (108 ± 3 mg/dl) in wild-type rats. Both metformin and sitagliptin alone had a comparable effect on restraining this increase in blood glucose concentration in HIP rats (increased to 154 ± 7 vs. 209 ± 12 mg/dl, P < 0.05), whereas the combination of sitagliptin and metformin had a synergistic effect (increased to 138 ± 8 vs. 209 ± 12 mg/dl, P < 0.01). Weight gain on the high-fat diet was comparable in wild-type (from 312 ± 5 to 628 ± 30 g) (Fig. 1*B*) and untreated HIP rats (from 291 ± 10 to 639 ± 14 g) (Fig. 1*B*) but was ~10% less in either sitagliptin- or metformin-treated HIP rats (P < 0.05) (Fig. 1*B*) and ~15% less in HIP rats treated with combination therapy (P < 0.05) (Fig. 1*B*). Food intake was decreased in HIP rats treated with metformin (~10%, P < 0.05 vs. HIP) (Fig. 1*C*) and with both metformin and sitagliptin (~20%, P < 0.05 vs. HIP) (Fig. 1*C*).

β-Cell mass, replication, and apoptosis. β-Cell mass was ~70% decreased in untreated HIP versus wild-type rats on high-fat diet (8.4 ± 1.3 vs. 25.6 ± 2.1 mg, P < 0.05) (Fig. 2) as a consequence of increased β-cell apoptosis, as previously reported (12). Sitagliptin therapy alone led to preservation of β-cell mass compared with untreated HIP rats (8.4 ± 1.3 vs. 16.6 ± 2.5 mg, P < 0.01) (Fig. 2). In HIP rats treated with metformin alone, β-cell mass was not significantly different from untreated HIP rats (8.4 ± 1.3 vs. 11.6 ± 1.3 mg, P = 0.24 for HIP rats vs. HIP rats given metformin) (Fig. 2), but those treated with combination therapy of sitagliptin and metformin had even better preservation of β-cell mass than those treated with sita-

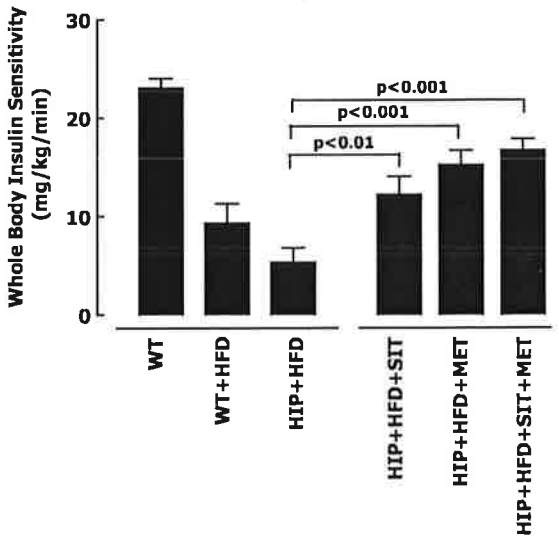

FIG. 4. Mean glucose infusion rates during the hyperinsulinemic-euglycemic clamp after 12-week treatment with 60% high-fat diet (HFD) in wild-type (WT) rats (n = 5), HIP rats (n = 6), HIP rats treated with sitagliptin (HIP+SIT; n = 7), HIP rats treated with metformin (HIP+MET; n = 6), and HIP rats treated with combination therapy (HIP+SIT+MET; n = 6). Data are means ± SE.

gliptin alone (8.4 ± 1.3 vs. 19.7 ± 2.4 mg, $P < 0.001$ for HIP rats vs. HIP rats given sitagliptin plus metformin) (Fig. 2).

The frequency of β-cell replication quantified by Ki-67 was increased by sitagliptin alone (0.2 ± 0.1 vs. 0.6 ± 0.1% for HIP rats vs. HIP rats given sitagliptin, $P < 0.05$) (Fig. 3*A* and *B*) and by combination therapy (0.2 ± 0.1 vs. 0.5 ± 0.1% for HIP rats vs. HIP rats given sitagliptin plus metformin, $P < 0.05$) (Fig. 3*A* and *B*). In contrast, metformin alone had no discernable effect on β-cell replication. Sitagliptin treatment alone decreased the frequency of β-cell apoptosis in HIP rats by ~55% ($P < 0.05$) (Fig. 3*C* and *D*). Metformin treatment alone was even more effective at decreasing β-cell apoptosis in HIP rats (by ~75%, $P < 0.05$) (Fig. 3*C* and *D*), whereas sitagliptin and metformin in combination had an action to suppress β-cell apoptosis that was comparable to that of metformin alone (Fig. 3*C* and *D*).

Insulin sensitivity. The impact of metformin and/or sitagliptin on insulin sensitivity was evaluated in HIP rats by hyperinsulinemic-euglycemic clamp and the isotope dilution technique. As expected, the high-fat diet induced insulin resistance in both wild-type and HIP rats (Fig. 4). Insulin sensitivity, assessed by the mean glucose infusion rates during the hyperinsulinemic period, was enhanced by either metformin (14.2 ± 1.4 vs. 5.3 ± 1.5 mg · kg^{-1} · min^{-1}, $P < 0.001$) (Fig. 4 and supplemental Table 1, which is available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0058/DC1) or sitagliptin (11.4 ± 1.7 vs. 5.3 ± 1.5 mg · kg^{-1} · min^{-1}, $P <$ 0.01) therapy alone compared with high-fat diet HIP rats, and in combination they had a slight additive effect (15.6 ± 1.1 vs. 5.3 ± 1.5 mg · kg^{-1} · min^{-1}, $P < 0.001$). In the fasting state, isotopically measured hepatic glucose release was approximately twofold greater in HIP versus wild-type rats (9.7 ± 1.4 vs. 5.1 ± 1.3 mg · kg^{-1} · min^{-1}, $P < 0.05$) (supplemental Table 1). In contrast, metformin alone or in combination with sitagliptin led to a ~40% suppression of fasting hepatic glucose release in HIP rats, whereas sitagliptin alone had no measurable effect on hepatic glucose release in the fasting state in HIP rats (supplemental Table 1). With insulin stimulation during the hyperinsulinemic-euglycemic clamp, hepatic glucose release in HIP rats was suppressed minimally compared with wild-type rats (7 vs. 100% for HIP vs. wild-type, $P < 0.05$) (supplemental Table 1), confirming marked hepatic insulin resistance. Metformin alone or in combination with sitagliptin partially restored hepatic insulin sensitivity in HIP rats, as indicated by ~60% suppression of hepatic glucose release during the hyperinsulinemic clamp (supplemental Table 1). Insulin-stimulated glucose disposal tended to be ~30% higher in all three drug-treated groups compared with nontreated HIP rats. The slightly decreased weight gain in the metformin- and sitagliptin-treated HIP rats may have contributed to the increased insulin sensitivity with each of these therapies.

β-Cell function. Glucose-mediated insulin secretion (examined by hyperglycemic clamp) was markedly attenuated in HIP compared with wild-type rats on a high-fat diet (648 ± 141 vs. 5,423 ± 480 pmol/l · min, $P < 0.05$) (Fig. 5*A*). There was no significant enhancement of first-phase (Fig. 5*A*) or second-phase (data not shown) glucose-mediated insulin secretion in HIP rats treated with sitagliptin or metformin alone when considered independently of insulin sensitivity. However, taking insulin sensitivity into account in the calculated disposition index (Fig. 5*B*), glucose-mediated insulin secretion in HIP rats was comparably enhanced by either metformin or sitagliptin alone, and in combination these drugs had a synergistic action to further enhance the disposition index ($P < 0.05$ for HIP rats vs. HIP rats given sitagliptin plus metformin) (Fig. 5*B*). Glucose-potentiated arginine-stimulated insulin secretion was also markedly attenuated in HIP versus wild-type rats (3,077 ± 528 vs. 8,809 ± 1,179 pmol/l, $P < 0.05$) (Fig. 5*C*), and again there was no appreciable benefit from either sitagliptin or metformin independently or in combination on this metric (Fig. 5*C*), which is generally considered a surrogate of β-cell mass (25). It is therefore of interest to note that the glucose-potentiated arginine-elicited first-phase insulin response did not reflect β-cell mass (Fig. 5*D*) in metformin- and/or sitagliptin-treated HIP rats.

Pancreatitis in an HIP rat treated with sitagliptin. The focus on the exocrine actions of sitagliptin arose as a consequence of an unexpected observation of marked necrotizing pancreatitis in one of eight rats treated with sitagliptin (Fig. 6 and supplemental Figs. 1 and 2). The region of pancreatitis was apparent as a mass of ~2 cm and histologically characterized by hemorrhagic necrosis, fibrosis, inflammatory cell infiltrate, and areas of ductal metaplasia (supplemental Figs. 1 and 2) (26–30). Although pancreatitis was present in 1 of 8 HIP rats treated with sitagliptin, it was not detected in any of the 17 HIP rats not treated with sitagliptin (Table 1) or any of the 89 HIP rat pancreases reported previously (7,10,12). Given this unexpected finding, we evaluated all the pancreases from this study for ductal metaplasia and increased ductal turnover, characteristics frequently present in pancreatitis in humans (29–31).

Ductal metaplasia. Ductal metaplasia was present in a total of three HIP rats treated with sitagliptin (Table 1). One of the three sitagliptin-treated HIP rats with ductal metaplasia also displayed marked pancreatitis (Table 1). These regions of ductal metaplasia were located both separate from and adjacent to islets of Langerhans (Fig. 7 and supplemental Fig. 3) and consisted of angulated

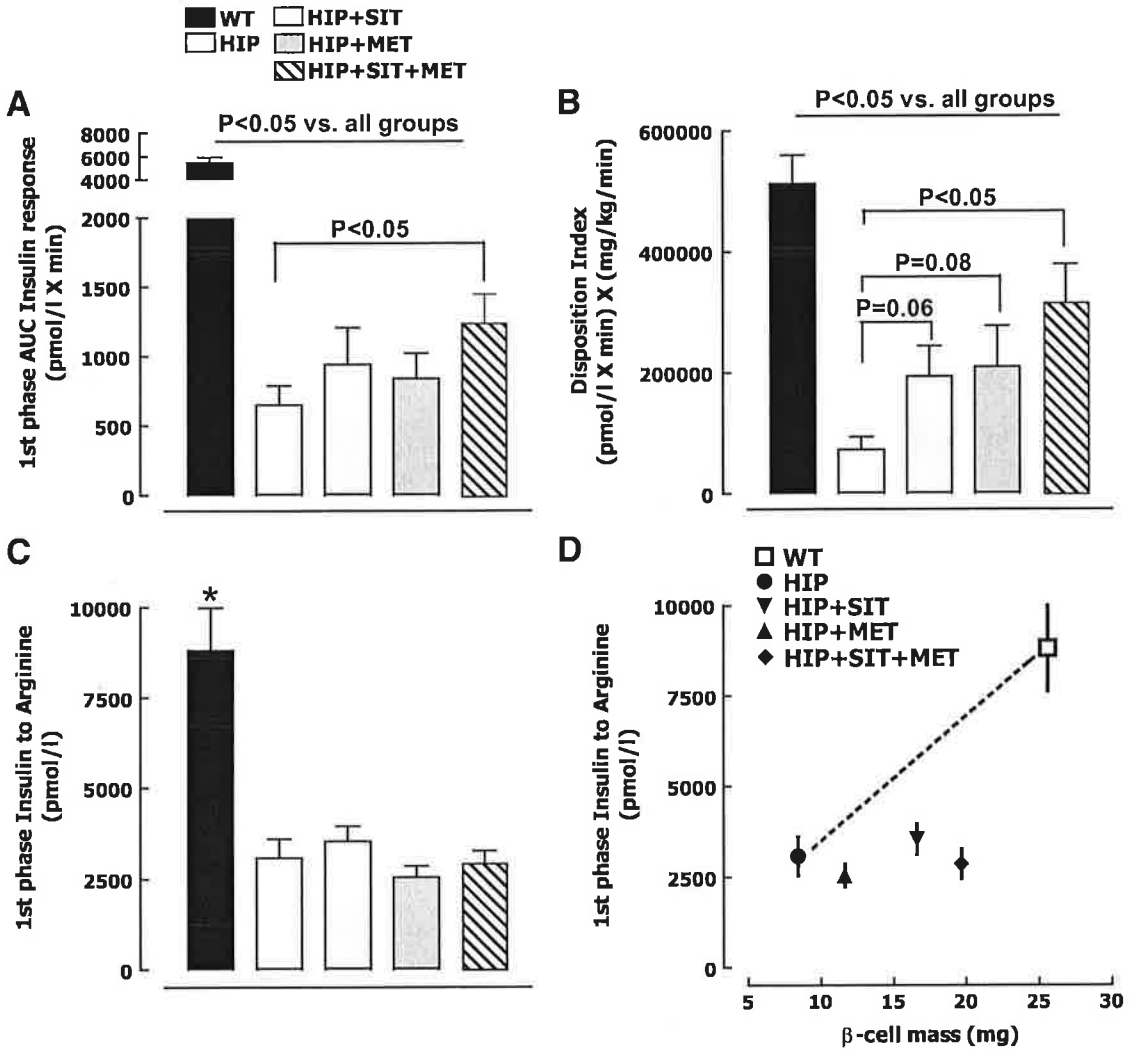

FIG. 5. Mean first-phase insulin response during the hyperglycemic clamp (*A*), mean disposition index (*B*), mean first-phase insulin response to arginine (*C*), and the relationship between β-cell mass and first-phase insulin response to arginine (*D*) after 12-week treatment with 60% high-fat diet in wild-type (WT) rats (n = 6), HIP rats (n = 8), HIP rats treated with sitagliptin (HIP+SIT; n = 8), HIP rats treated with metformin (HIP+MET; n = 6), and HIP rats treated with combination therapy (HIP+SIT+MET; n = 6). Data are means ± SE.

tubular structures, interspersed fibrosis, and inflammatory cells. In some areas these were adjacent to atrophic acinar cells (Fig. 7 and supplemental Fig. 3). Ductal metaplasia was immunoreactive for cytokeratin and Ki-67 (Fig. 8*A*), indicating a high rate of cell turnover. Furthermore, metaplastic areas included numerous fibroblasts (by morphology and fibrinectin immunoreactivity) (Fig. 8*B*) and were absent of PDX-1 expression (Fig. 8*C*).

Ductal cell turnover. Ductal replication quantified by Ki-67 immunoreactivity was increased fourfold in untreated diabetic HIP rats versus wild-type controls (0.6 ± 0.2 vs. 2.5 ± 0.3%, $P < 0.05$) (Figs. 9 and 10*A*). Sitagliptin treatment led to an additional three-fold increase in the frequency of ductal cell replication versus untreated HIP rats (2.5 ± 0.3 vs. 7.3 ± 0.7%, $P < 0.05$) (Figs. 9 and 10 and supplemental Fig. 4) and a 12-fold increase compared with wild-type rats. Intriguingly, metformin treatment abrogated the effects of sitagliptin on ductal cell proliferation (7.3 ± 0.7 vs. 1.4 ± 0.6%, $P < 0.05$ for HIP rats given sitagliptin vs. HIP rats given sitagliptin plus metformin) (Figs. 9 and 10*A*). The frequency of ductal replication was positively correlated with fasting blood glucose concentration, with an apparent continuum between wild-type and HIP rats (Fig. 10*C*), with the sitagliptin-only–treated group displaced to a higher slope (Fig. 10*C*). Addition of metformin to sitagliptin restored the frequency of ductal replication to the same relationship with fasting glucose concentrations observed in rats not exposed to sitagliptin (Fig. 10*C*).

GLP-1 receptor, PDX-1, and insulin expression in exocrine ducts. As previously reported (32), GLP-1 receptors were expressed in pancreatic ducts, but with no differences between treatment groups (supplemental Fig. 5). PDX-1– and insulin-positive ductal cells were observed after sitagliptin treatment in HIP rats (supplemental Fig. 6*A*). Although most β-cells were present within well-defined islets, occasional individual β-cells were present scattered in the exocrine pancreas. These scattered β-cells

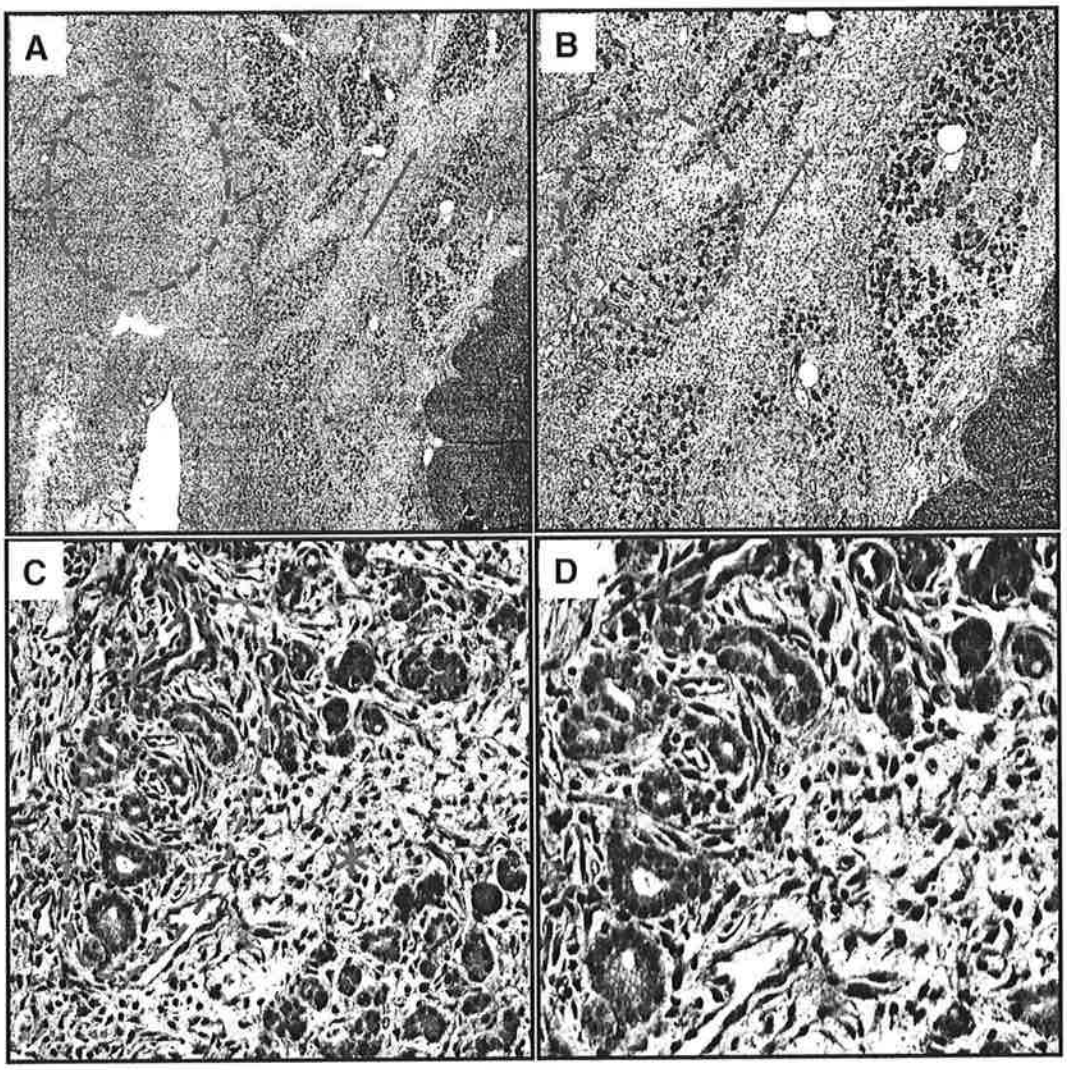

FIG. 6. Necrotizing pancreatitis in a HIP rat treated with sitagliptin for 12 weeks. *A*: Representative image at ×2 magnification of the exocrine pancreas stained for hematoxylin and eosin from an HIP rat treated with sitagliptin for 12 weeks with necrotizing pancreatitis. Note partially preserved lobular configuration of the exocrine pancreas; however, note the significant loss of acinar cell density and the widening of the septae (arrow) as well as a complete loss of acinar cells in some areas (circle). *B*: Representative image at ×4 magnification. At this higher magnification, septal fibrosis and inflammation (arrows) are better appreciated as well as partial and complete loss of acinar cells (circle). *C*: Representative image at ×20 magnification. At this magnification, acinar cell injury and angulated tubular ductal structures within the acini are clearly seen (circle). Note the extensive septal inflammation and fibrosis (*). *D*: Representative image at ×40 magnification. At this higher magnification, angulated tubular ductal structures and surrounding tissue fibrosis are better appreciated. (A high-quality digital representation of this figure is available in the online issue.)

were approximately sixfold more abundant in sitagliptin-treated compared with untreated HIP rats ($P < 0.05$) (supplemental Fig. 6*B*). Interestingly, the number of scattered β-cells was also increased in metformin-treated animals, but not in animals that received combination therapy of sitagliptin plus metformin.

TABLE 1
Incidence of pancreatitis, ductal metaplasia, and increased ductal turnover by group

	Wild type	HIP	HIP + SIT	HIP + MET	HIP + SIT + MET
Number studied	7	8	8	9	8
Pancreatitis	0	0	1	0	0
Ductal metaplasia	0	0	2	0	1
Increased ductal proliferation*	—	4	8	2	1

Data are *n*. *Increase in ductal cell proliferation was defined as an increase in ductal proliferation 3 SDs above the mean of wild-type rats. HIP + MET, HIP rats treated with metformin; HIP + SIT, HIP rats treated with sitagliptin; HIP + SIT + MET, HIP rats treated with combination therapy.

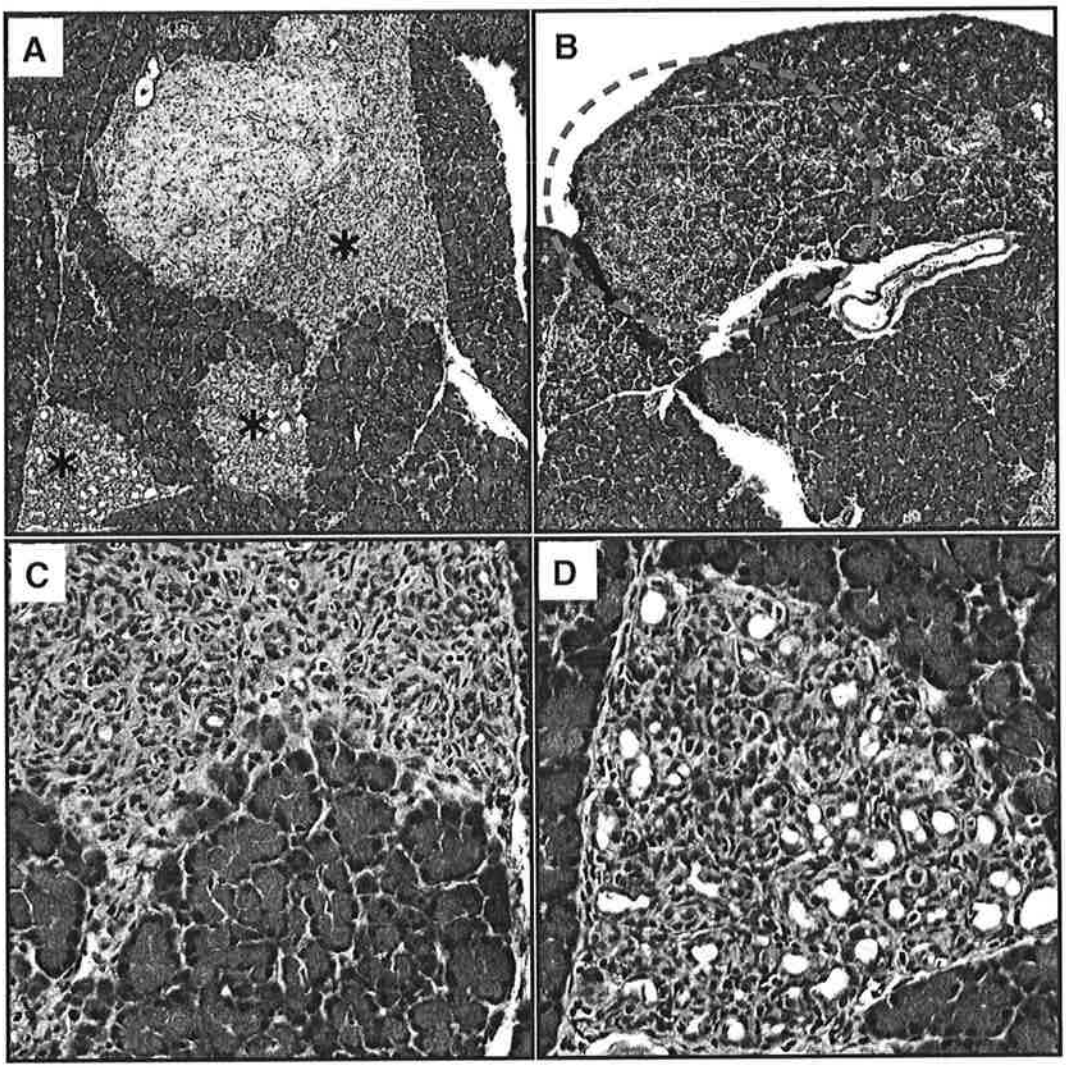

FIG. 7. Extensive ductal metaplasia in HIP rats treated with sitagliptin for 12 weeks. *A* and *B*: Representative images at ×10 magnification of ductal cell metaplasia observed in a rat treated with sitagliptin for 12 weeks. Metaplastic regions consisted of angulated tubular structures, interspersed fibrosis, and inflammatory cells and were located both adjacent to islets of Langerhans (*) as well as separated from islets (circle). *C*: Representative image at ×20 magnification. At this higher magnification, an apparent transition from intact acinar cells to damaged/atrophic acinar cells to angulated tubular ductal structures is seen. *D*: Representative image at ×40 magnification. At this magnification the presence of extensive angulated tubular ductal structures and surrounding tissue fibrosis within the metaplastic region is better appreciated. (A high-quality digital representation of this figure is available in the online issue.)

DISCUSSION

Our primary objective was to establish whether metformin or sitagliptin alone and in combination favorably modified disease progression in the HIP rat model of type 2 diabetes. Although loss of β-cell mass in the HIP rat was slowed by this combination therapy, unexpected adverse actions on the exocrine pancreas were also observed.

Metformin has been shown to delay type 2 diabetes onset in humans (13). Because enhanced insulin sensitivity through lifestyle changes also delays diabetes (13), at least part of the protective effect of metformin may be mediated by metformin's actions to enhance hepatic insulin sensitivity through its actions on AMP-activated kinase (33). Metformin decreased β-cell apoptosis in isolated human islets from patients with type 2 diabetes (34). In the current study, metformin was more effective than sitagliptin in reducing β-cell apoptosis in the high-fat diet –fed HIP rat. Although sitagliptin alone also suppressed β-cell apoptosis, there was no added benefit of sitagliptin on metformin-mediated suppression of β-cell apoptosis. Sitagliptin enhanced β-cell replication in HIP rats, consistent with prior studies of GLP-1– and GLP-1 mimetic–induced β-cell replication in a variety of murine models (14,15,32). The benefits of sitagliptin and/or metformin on β-cell mass and function reported here may have been mediated by either direct effects of the drugs on β-cells or indirectly by their actions to lower blood glucose. Hyperglycemia can contribute to both loss of β-cell mass by increasing β-cell apoptosis and/or loss of β-cell function (35). The current study was designed to examine effects of sitagliptin and metformin treatment in an in vivo model of type 2 diabetes, with the advantage of best approaching actions in humans with type 2 diabetes, but with the

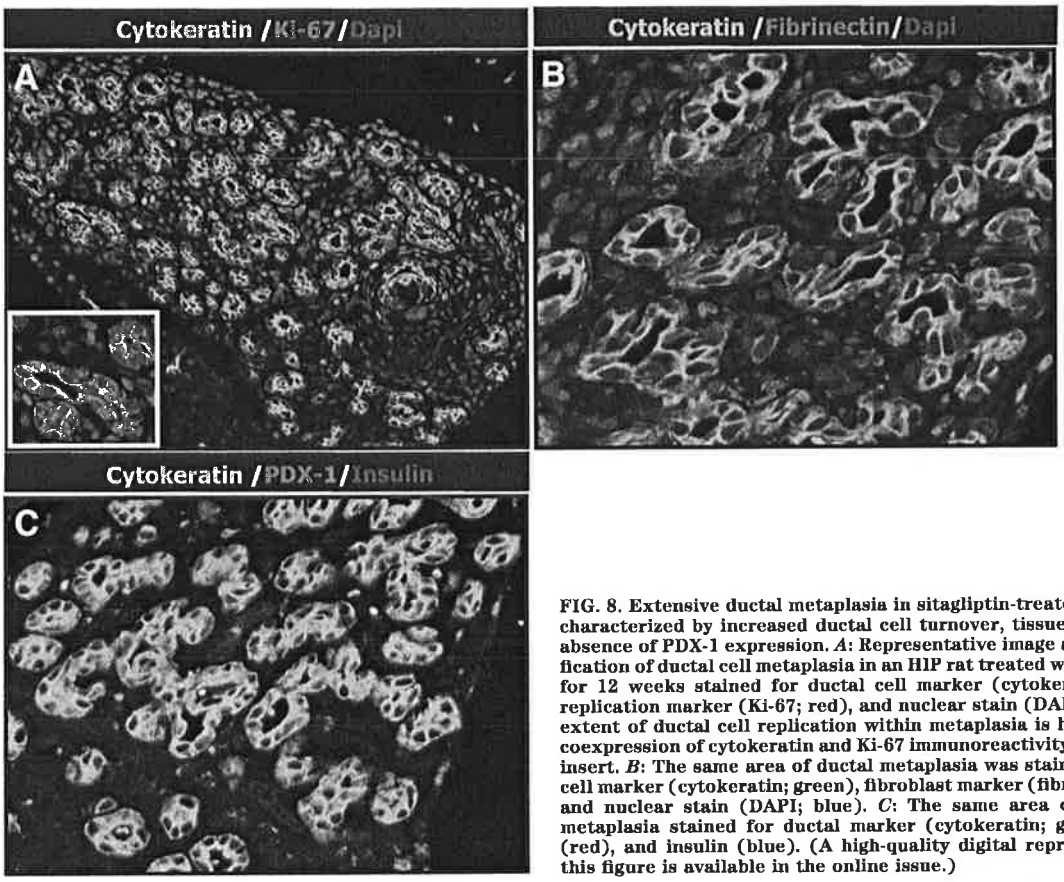

FIG. 8. Extensive ductal metaplasia in sitagliptin-treated HIP rats is characterized by increased ductal cell turnover, tissue fibrosis, and absence of PDX-1 expression. *A*: Representative image at ×20 magnification of ductal cell metaplasia in an HIP rat treated with sitagliptin for 12 weeks stained for ductal cell marker (cytokeratin; green), replication marker (Ki-67; red), and nuclear stain (DAPI; blue). The extent of ductal cell replication within metaplasia is highlighted by coexpression of cytokeratin and Ki-67 immunoreactivity shown in the insert. *B*: The same area of ductal metaplasia was stained for ductal cell marker (cytokeratin; green), fibroblast marker (fibrinectin; red), and nuclear stain (DAPI; blue). *C*: The same area of ductal cell metaplasia stained for ductal marker (cytokeratin; green), PDX-1 (red), and insulin (blue). (A high-quality digital representation of this figure is available in the online issue.)

limitation of precluding distinction between direct and indirect effects of drugs on β-cell mass and function.

GLP-1–mediated increased β-cell replication has to be interpreted with caution. Juvenile rodents, in common with juvenile humans, have a period of postnatal expansion of β-cell numbers mediated by β-cell replication (36,37). Such studies (including this one) in relatively young rodents have exposed β-cells to increased GLP-1 when they remain replication competent. Recent studies have demonstrated that the capacity for new β-cell formation through β-cell replication is attenuated in adult rodents after epigenetic modifications of β-cells, and thus, not surprisingly, older rodents do not exhibit the same GLP-1–mediated β-cell replication as that observed in juvenile rodents (38,39). It is perhaps not surprising that under conditions of increased GLP-1 secretion (post–gastric bypass) in humans, despite earlier predictions (40), neither β-cell replication nor the fractional area of pancreas occupied by β-cells was increased (41). Likewise, long-standing exposure of nonhuman primates to the GLP-1 mimetic exenitide was reported to not increase β-cell mass (42). Because the incremental effect of sitagliptin on metformin to preserve β-cell mass in the current study appeared to be mediated through its action to foster β-cell replication, it is possible that no such added benefit would be present in humans.

The unexpected finding of hemorrhagic pancreatitis in one of the sitagliptin-treated rats prompted further analysis of the exocrine pancreas in this study. We report increased ductal proliferation in all sitagliptin-treated rats that were not also treated with metformin. We also noted ductal metaplasia in three sitagliptin-treated rats, one of which was also treated with metformin. Increased ductal proliferation and ductal metaplasia are well-recognized components of pancreatitis in humans (29–31), and they therefore offer a plausible mechanism for the GLP-1–induced pancreatitis reported in humans treated with the GLP-1 mimetics exenatide or liraglutide (22,23). Ductal GLP-1 receptor expression was not altered in any of the treatment groups. It cannot be assumed that the actions of sitagliptin to induce exocrine (or endocrine) pancreatic changes are mediated through GLP-1 because other regulatory peptides are also degraded by DPP-4 (43). Furthermore, we cannot rule out direct actions of sitagliptin on the exocrine pancreas. However, because pancreatitis has also been reported in humans treated with GLP-1 agonists (22,23), it seems likely that the exocrine effects of sitagliptin treatment reported here are a consequence of increased GLP-1 concentrations.

Perhaps of most concern, increased ductal cell turnover and ductal metaplasia are also well-characterized risk factors for pancreatic ductal cancer (31,44,45), as is pancreatitis (46). As yet, no increase in pancreatic cancer has been reported in patients treated with GLP-1 mimetics or DPP-4 inhibitors. However, these drugs have only been available for a relatively short period of time. Any influence that GLP-1–based therapy might have to increase the incidence of pancreatic cancer through chronically increased ductal cell turnover would be expected to take several years. The incidence of colon carcinoma associated with chronic epithelial replication and regeneration in

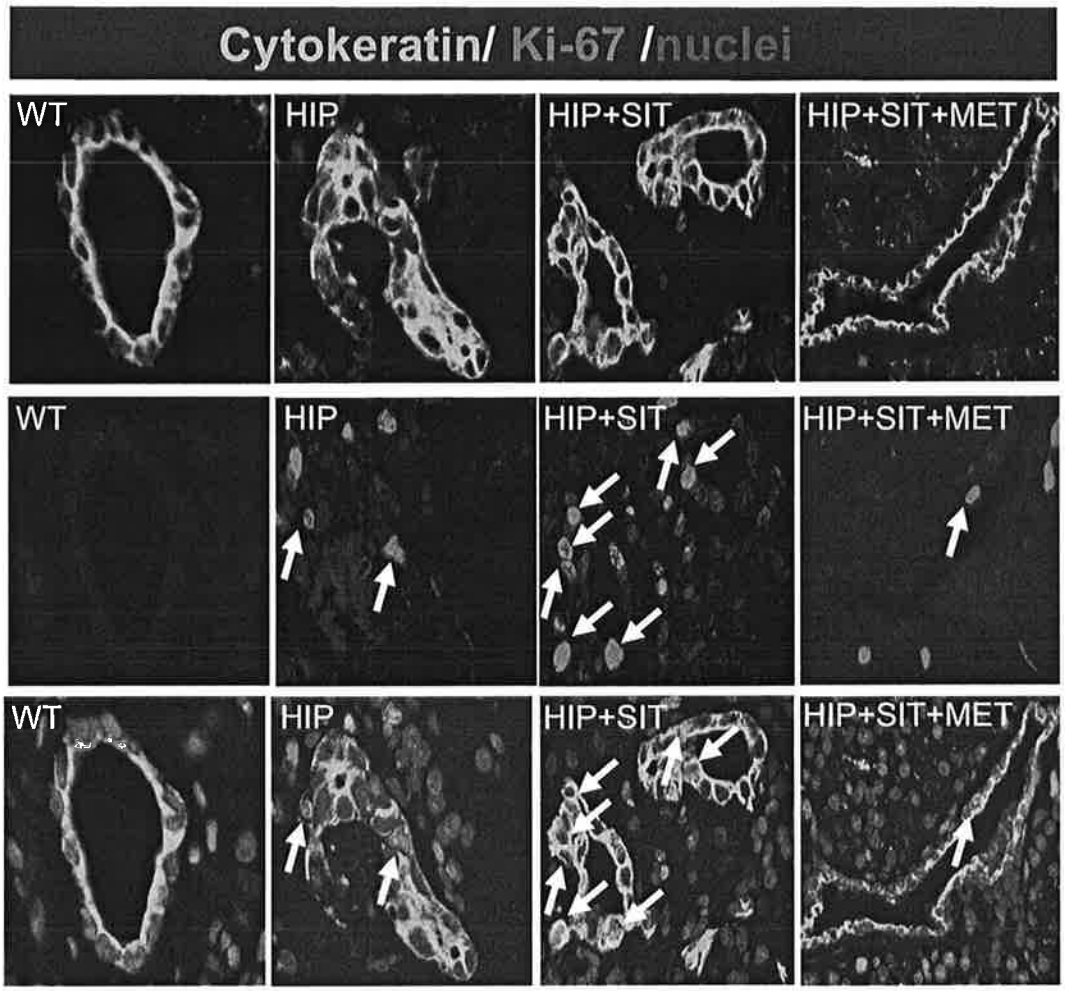

FIG. 9. Ductal cell replication is increased in HIP rats treated with sitagliptin for 12 weeks. Representative images at ×20 magnification of exocrine ducts stained for cytokeratin (green), replication marker Ki-67 (red), and nuclear stain (DAPI; blue) in wild-type control rats, diabetic HIP rats, HIP rats treated with sitagliptin, or HIP rats treated with combination therapy. *Examples from sitagliptin-treated rats represent metaplasia and pancreatitis-free areas of the exocrine pancreas. Arrows indicate cytokeratin/Ki-67–positive cells. (A high-quality digital representation of this figure is available in the online issue.)

the setting of inflammation in ulcerative colitis starts to increase 8–10 years after disease onset (47).

The current study may also shed some light on the increased incidence of pancreatitis and pancreatic cancer in patients with diabetes. Although pancreatitis or pancreatic cancer can lead to diabetes (48), epidemiological studies imply that the converse may also be true (49–52). Exocrine pancreatic insufficiency and pancreatitis are common in both autoimmune-mediated type 1 and type 2 diabetes (48,53). In the current study, we noted increased ductal turnover in the HIP rat related to plasma glucose concentrations (Fig. 10*C*). This implies that hyperglycemia per se may be sufficient to induce increased ductal cell turnover. While controversial, it has been proposed that there may be attempted β-cell regeneration in diabetes from progenitor cells that are proximate to, or within, pancreatic ducts (54).

Moreover, it has been proposed that GLP-1–based therapy enhances β-cell formation by increasing β-cell transdifferentiation from these putative duct-related stem cells (14,32). The action of sitagliptin treatment alone to increase ductal replication, apparently still in a glucose-sensitive manner but at a higher set point, is consistent with a complimentary interaction between glucose and GLP-1 concentrations to activate ductal cell proliferation. The observed PDX-1– and insulin-positive ductal cells in sitagliptin-treated HIP rats support this postulate.

An intriguing finding in the current study is the fact that addition of metformin to sitagliptin prevented the sitagliptin-mediated increase in ductal replication. Because metformin therapy has been shown to increase GLP-1 levels in some studies (55), the action to counter sitagliptin-mediated increased ductal replication is presumably independent of GLP-1. It is possible that the effect was mediated indirectly through metabolic actions of metformin to enhance insulin sensitivity or decrease blood glucose concentrations (56). Alternatively, metformin might act directly on ductal cells to suppress proliferation. Antiproliferative effects of metformin have been reported in prostate cancer cell lines and explanted prostate cells in mice (57). Recent epidemiological studies have revealed that metformin therapy is associated with a reduced incidence of cancer, including pancreatic cancer (58). The

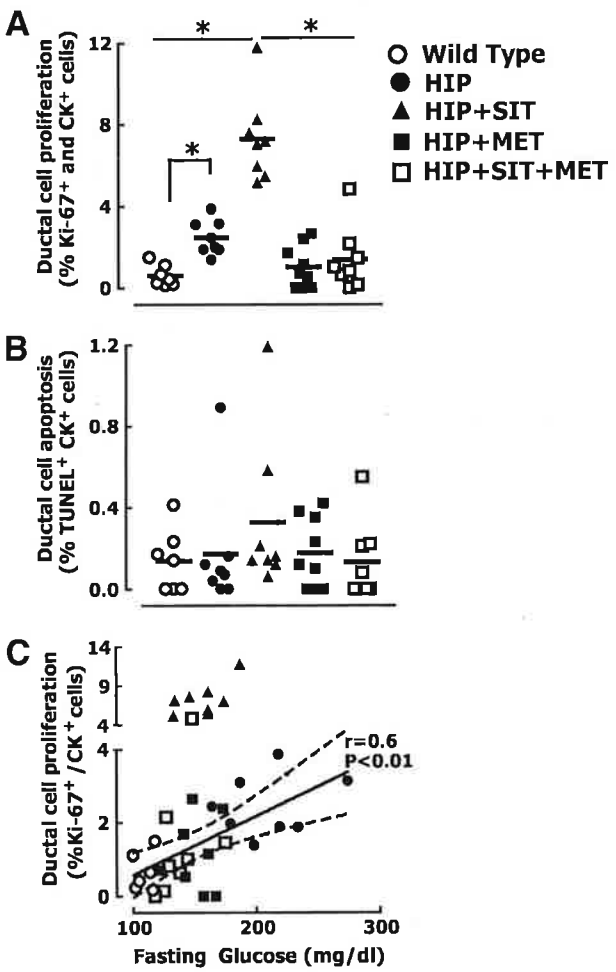

FIG. 10. Increased ductal cell turnover in HIP rats treated with sitagliptin. Quantification of ductal cell replication (*A*) and apoptosis (*B*) in wild-type rats, HIP rats, and HIP rats treated with either sitagliptin (HIP+SIT), metformin (HIP+MET), or combination therapy of sitagliptin and metformin (HIP+SIT+MET) for 12 weeks. *C*: Regression analysis of the relationships between ductal cell proliferation versus fasting plasma glucose. Note that ductal cell replication in sitagliptin-treated rats was quantified only in metaplasia and pancreatitis–free areas of the exocrine pancreas. *$P < 0.05$.

latter might be a consequence of the metabolic actions and/or the direct antiproliferative effects of metformin.

It is unknown whether sitagliptin actions on ductal turnover and/or induction of ductal metaplasia observed in the HIP rat extends to humans. It is plausible that these effects are restricted to the rat. It will be important to address this in pancreata, when available, from humans with type 2 diabetes who have been treated with GLP-1 mimetic therapy. Because the action of sitagliptin to increase ductal turnover was dependent on hyperglycemia, GLP-1 mimetic treatment on the exocrine pancreas in nondiabetic animal models, as used in classical toxicology screening studies, would presumably miss this effect and its potential long-term adverse consequences.

In summary, sitagliptin, and metformin, had synergistic effects on preserving β-cell mass in the HIP rat model of type 2 diabetes. Metformin was most effective at suppressing β-cell apoptosis. Sitagliptin fostered increased β-cell replication, but this is likely of limited benefit in adult humans. Of concern, we noted pancreatitis in one, ductal metaplasia in three and increased ductal turnover in all sitagliptin-treated HIP rats. Because the apparent adverse effects of GLP-1 mimetic therapy are at least to some extent offset by concurrent use of metformin, it is perhaps judicious to use GLP-1 mimetic therapy (including DPP-4 inhibiters) only in addition to metformin until potential long-term adverse effects of GLP-1–based therapy on exocrine pancreas can be ruled out in humans with diabetes.

ACKNOWLEDGMENTS

Studies were supported by grants from the National Institutes of Health (NIH) (DK59579) and the Larry Hillblom Foundation (to P.C.B.). A.V.M. was supported by the NIH Ruth L. Kirschstein National Research Service Award.

Studies were also supported by the Merck Research Foundation (to P.C.B.). No other potential conflicts of interest relevant to this article were reported.

Parts of this study were presented in abstract form at the 69th Scientific Sessions of the American Diabetes Association, New Orleans, Louisiana, 5–9 June 2009.

We are grateful for the support and excellent suggestions of our colleagues at the Larry Hillblom Islet Research Center, Anil Bhushan and Senta Georgia, and are also grateful to Matthias Hebrok from the University of California San Francisco.

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